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* This series of papers is the result of a conference on *Fourth Tissue Homotransplantation Conference* held by The New York Academy of Sciences, on February 4, 5, and 6, 1960.

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INTRODUCTORY REMARKS

John M. Converse and Blair O. Rogers
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Two important additions were made to the Fourth Tissue Homotransplantation Conference on which this monograph is based that were not included in the previous conferences:[1,2,3] the first was the introduction of a session dealing exclusively with organ transplantation, and the second was the recording of the discussion that followed each paper. We hope the reader will agree with us that the publication of the discussions of the papers adds much to the scientific interest of this monograph.

Another innovation is the early date of publication of this monograph within four months after the conference. The contributors of papers must be thanked for their willingness to submit to the deadlines made necessary for early publication. The rapid march of progress in tissue homotransplantation makes it imperative that recent developments be made available to all workers in the field as early as possible. The advances reported in this monograph and those that will surely arise within the next two years prior to the fifth biennial conference can do nothing but increase our optimism for the future of transplantation biology, whether it deals with the transplanting of isolated tissues or even of a complex combination of tissues and organs. If we may borrow from the ever stimulating remarks of our Scottish colleague Michael Woodruff,[4] made in a recent review of the surgery of replacement, a fitting way to end these introductory remarks would be to quote the final portion of his review: "It may well be . . . that we shall succeed in devising methods of making tissues and organs from one human being survive permanently in another, not only when there is some special relationship between donor and host but as a general rule. If so, we shall stand on the threshold of a new era in surgery and we shall have found a new meaning for that excellent motto *Nemo sibi nascitur*—"No man is born for himself alone."

References

1. Annals of The New York Academy of Sciences. 1955. **59**(3): 277—466.
2. Annals of The New York Academy of Sciences. 1956. **64**(5): 735—1073.
3. Annals of The New York Academy of Sciences. 1958. **73**(3): 539—868.
4. Woodruff, M. F. A. 1957. J. Roy. Coll. Surg. Edinburgh. **3**: 19.

THE EVOLUTION OF TISSUE HOMOTRANSPLANTATION RESEARCH

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In 1954 The New York Academy of Sciences published a monograph entitled *The Relation of Immunology to Tissue Homotransplantation*,[1] based on a conference convened under its sponsorship. Since then monographs on tissue homotransplantation have been published by the academy biennially, based on subsequent conferences on the subject held at two-year intervals.[2,3] Of primary concern to contributors to the first of these monographs were problems of definition and descriptions of the phenomena of transplant rejection. This publication also contained the last gasps of the "nurture" versus "nature" concept in tissue homotransplantation, and the growth of interest in possible immunological causes of homograft rejection reactions.

This first monograph, whose highlights are summarized in TABLE 1, was representative of what we have termed the period of definition in homotransplantation research. Rejection patterns of transplants of skin, bone, cornea, ovaries, and kidneys were described. Only moderate attention was given to two additional areas reported at this conference: the search for mechanisms of rejection and the study of altered patterns of homograft reactivity. For this reason, the most noteworthy contribution to this conference, the report by Billingham *et al.*[4] of the induction of acquired tolerance to skin homografts in chickens, rabbits, and mice, aroused relatively little attention. The enhancement phenomenon, another landmark in homograft research, was also described in this monograph, but also evoked only a moderate reaction.

As new methods of study and objective criteria for the evaluation of homograft rejection were developed, the focus of investigation now turned to the problem of mechanisms. This second phase, which we have termed the period of growth, was reflected in the contributions to the second monograph, which are outlined in TABLE 2. It is noted that three main lines of approach were followed in the attempt to study mechanisms of homograft rejection. These included (1) a search for the actual mechanisms of homograft rejection, employing chiefly the "circulating antibody" and the "delayed hypersensitivity" hypotheses. The occurrence of immune sera to transplanted mouse tumors seemed to favor the former idea, while the description of the recall flare in man and the results of studies with the Algire chamber in mice supported the latter; (2) a study of altered reactivity patterns, in terms of man-made alterations in the recipients, including burns, irradiation, or antigenic pretreatment, or in actual disease states (particularly agammaglobulinemia and uremia), or in terms of the induction of tolerance (a concept that now finally had come into its own); and (3) a phylogenetic search for homograft rejection patterns in lower animal species, in an attempt to gain a clearer understanding of these reactions in humans.

In the two years following publication of the second monograph, an impressive array of new data accumulated. However, as noted in TABLE 3, which summarizes the contents of the third monograph, most of the contributors were concerned with the confirmation and extension of existing concepts, largely following the outlines appearing in the previous publications. Our knowledge of homograft rejection patterns was broadened, and further contributions were made to the study of mechanisms of rejection. For this reason, we have termed this third phase of research in tissue homotransplantation the period of consolidation.

---

* Lincoln Ellsworth Research Fellow.

One of the causes of the rapid growth of interest in and knowledge of tissue

TABLE 1
FIRST CONFERENCE: THE PERIOD OF DEFINITION

I. "Nurture" versus "nature" concept
II. Immunology as a discipline for the study of tissue transplantation
  (1) Definition of tissue rejection patterns
    (a) Skin homografts
    (b) Bone homografts
    (c) Corneal homografts
    (d) Ovarian homografts
    (e) Kidney homografts
  (2) Search for mechanisms of rejection
    (a) Possibility of circulating antibodies
  (3) Study of altered reactivity patterns
    (a) Artificial alterations
    (b) Enhancement
    (c) Tolerance

TABLE 2
SECOND CONFERENCE: THE PERIOD OF GROWTH
Immunology as a Discipline for the Study of Tissue Homotransplantation

I. Definition of tissue rejection patterns
  (1) Kidney
  (2) Lymph node cells
II. Search for mechanisms of rejection
  (1) Circulating antibody hypothesis
    (a) Isoimmune and cytotoxic sera in mouse cancers
  (2) Delayed hypersensitivity theory
    (a) The recall flare
    (b) Algire chamber studies
    (c) Histopathology of the regional draining lymph nodes in rejection reaction
III. Study of altered reactivity patterns
  (1) Artificial alterations
    (a) Burns
    (b) X rays
    (c) Pretreatment with epidermal cells
  (2) Enhancement
    (a) Antigens involved
    (b) Antibodies or factor concerned
  (3) Tolerance
    (a) Postpartum induction in rats
    (b) Relation to inhibition of antibody formation
  (4) Disease states
    (a) Agammaglobulinemia
    (b) Uremia
IV. Phylogenetic analysis and genetic analysis
  (1) Homograft rejection in the goldfish
  (2) Homograft survival and relationship to sex linkage in mice
  (3) Relationship between genetic factors and skin homograft survival in humans

homotransplantation has been the intimate cooperation, over several years, of individuals representative of a multitude of different scientific disciplines, including biochemistry, immunology, genetics, pathology, and physiology, as well as general medicine and zoology. During this early period of development, research workers in the field of tissue transplantation borrowed from each of these fields. However, as laboratory experience in tissue transplantation accumulated, these borrowed techniques and methods soon became accepted procedure in this new science of tissue transplantation. Gradually a new composite body of knowledge and methodology arose, and workers in tissue transplantation research were no longer specialists of only one mode of experimental study; rather, they became trained in a great variety of highly specialized laboratory techniques. They became biologists in every sense of the word. Indeed, as knowledge and understanding of tissue transplantation phenomena grew, orig-

TABLE 3

THIRD CONFERENCE: THE PERIOD OF CONSOLIDATION

I. Definition of tissue rejection patterns
    (1) Role of ground substance
    (2) Fetal tissues
    (3) Bone marrow
    (4) Tooth buds
    (5) Lymph node cells
    (6) Cancer cells in man
    (7) Effect of vascularization
II. Search for mechanisms of rejection
    (1) Circulating antibodies
        (a) H-2 antibody
        (b) Serum transfer of graft rejection
    (2) Cellular hypersensitivity
        (a) Role of lymphatics
        (b) Cell versus host reactions
    (3) Antigens
            Tissue antigens in
            (a) Autosensitization
            (b) Skin-homograft rejection
            (c) Aspermatogenesis
III. Study of altered reactivity patterns
    (1) Artificial alterations
        (a) X rays alone or with bone marrow
        (b) Protein deficiency
        (c) Desensitization of adult
        (d) Guinea pigs (delayed hypersensitivity)
    (2) Enhancement
        (a) Whole blood
    (3) Tolerance
        (a) In the rat
        (b) In the chicken
        (c) To nerve tissue
        (d) Embryonic tissue
IV. Phylogenetic and genetic analysis
    (1) Comparative phylogeny of homotransplantation
    (2) Histocompatibility
        (a) Humans
        (b) Inbred mice
        (c) Inbred guinea pigs
        (d) Fishes

inal roles were reversed and, today, this new broad biological discipline has made valuable contributions to the specialties that had supplied it with its original technology and methodology.

The data presented in the present monograph clearly illustrate this progression of events. This volume marks the emergence of tissue homotransplantation research as a specialized discipline particularly adapted to the study of the broad patterns of normal and altered biological activity, with special reference to the problems of allergy, malignancy, and parturition. Many of the reports have an important bearing on these problems and will arouse considerable discussion, as well as stimulate further research. We conclude this brief review with a proposal: that the new discipline whose rise is symbolized in these pages be termed transplantation biology.

References

1. ANNALS OF THE NEW YORK ACADEMY OF SCIENCES. 1955. The relation of immunology to tissue homotransplantation. **59**(3): 277–466.
2. ANNALS OF THE NEW YORK ACADEMY OF SCIENCES. 1956. Second tissue homotransplantation conference. **64**(5): 735–1073.
3. ANNALS OF THE NEW YORK ACADEMY OF SCIENCES. 1958. Third tissue homotransplantation conference. **73**(3): 539–868.
4. BILLINGHAM, R. E., L. BRENT & P. B. MEDAWAR. 1955. Acquired tolerance of skin homografts. Ann. N. Y. Acad. Sci. **59**(3): 409.

Part I. Immunogenetics and Phylogenetics

DOSAGE AND ADDITIVE EFFECTS OF HISTOCOMPATIBILITY GENES IN THE TELEOST *XIPHOPHORUS MACULATUS**

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INTRODUCTION

The rejection or acceptance of a graft depends primarily upon histocompatibility genes. Tissue transplanted between monozygous twins, among members of highly inbred strains, or among members of a clone that reproduce exclusively by parthenogenesis or gynogenesis (Kallman, 1960) will survive, because host and donor possess identical genotypes. Transplants among unrelated hosts and donors will be rejected, since they possess different histocompatibility genes. These genes are thought to be related to the production of specific antigens. Hence the presence of "foreign" antigens in a graft may lead to the production of antibodies that act upon the graft and destroy it. This concept was derived largely from experiments with inbred strains of rats and mice.

Either each histocompatibility gene gives rise to a specific antigen in large quantities, so that differences between the homozygous and heterozygous conditions are unimportant, at least as far as the antigenic content of the graft is concerned, or each gene gives rise to only a limited quantity, so that appreciably more is produced in the homozygous than in the heterozygous condition. Differences in antigenic level have been investigated in transplantation studies involving two inbred strains and their F_1 hybrids, where interstrain grafts (for example, from *AA* donors to *aa* hosts; *A* and *a* representing different alleles at the same histocompatibility locus) possess twice as many foreign histocompatibility genes as do F_1 grafts (*Aa*). Differences in survival times of heterozygous and homozygous grafts may therefore reflect differences in gene dosage.

Billingham *et al.* (1954) and Barnes and Krohn (1957) found no differences between the survival times of homozygous and heterozygous transplants in mice, but Prehn and Main (1954) observed an apparent dosage effect. Breyere (1957) reported a dosage effect for histocompatibility genes in the rat. The existence of such an effect was indicated clearly in the experiments of Kallman and Gordon (1958), who found that dorsal and anal fins of the platyfish *Xiphophorus maculatus* transplanted from the F_1 hybrids of two inbred strains into hosts of either parent strain (P_1) survived consistently longer than interstrain grafts.

The dosage effect was investigated herein by making transplants from homozygous (P_1) and heterozygous (F_1) donors into hosts of various genotypes. The following questions were explored:

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* This paper is taken from a dissertation submitted to the Graduate School of Arts and Science, New York University, New York, N. Y., in partial fulfillment of the requirements for the degree of Doctor of Philosophy. The work reported was supported in part by Grant C-297 from the National Cancer Institute, Public Health Service, Bethesda, Md., to the late Myron Gordon. It was aided further by the laboratory facilities of the American Museum of Natural History, New York, N. Y.

(1) How many histocompatibility genes are involved in determining the fate of transplants made between members of two inbred strains of platyfish?

(2) If host and donor differ at only a single histocompatibility locus, will the assumed slight antigenic difference be sufficient to cause a homograft reaction in both homozygous and heterozygous grafts?

(3) What is the relationship between the survival time of a graft and the genetic propinquity of host and donor?

(4) How does previous immunization of the host by a primary homograft affect the survival time of a secondary P_1 or F_1 graft?

MATERIALS AND METHODS

Origins of Inbred Strains

Since its collection in 1939, strain 30 has been inbred by brother-sister matings for 25 generations. Strain 163, which was derived from the same sample, has been inbred by brother-sister matings for 12 generations. These 2 strains are now quite distinct and differ most noticeably in their pigment patterns. The F_1 generation of the cross $30^{23} \times 163^{11}$ (the superscript numbers indicate the inbred generations) is represented by pedigree 784, their F_2 generation by 793, and their F_3 generation by pedigree 850. The F_1 generation of the cross $30^{24} \times 163^{12}$ is represented by pedigree 859. The backcross generation (Bc) of F_1 (784) $\times$ 163^{12} is represented by pedigree 816, and F_1 (784) $\times$ 30^{24} by pedigree 847.

Transplantation Techniques

Kallman and Gordon (1957, 1958) described a method for the transplantation of dorsal and anal fins that involved the insertion of the fin's suspensorium into a slit or pocket that was prepared in the musculature of the host (FIGURE 1). Essentially the same procedure has been adopted for the tail fin. Due to the bulk of this structure as compared with anal and dorsal fins, it is necessary to make an unusually large incision that extends over three fourths of one side of the host. A wound of such size invariably leads to the death of the host or to loss of the graft. This operation is greatly facilitated by using as hosts fish that are slightly larger and older than the donors.

The tail fin is amputated from the caudal peduncle by making a cut at the level of the urostyle. This procedure leaves intact the fin rays and the hypural and epural bones with which they articulate. These bones are squeezed into the pocket, thus anchoring the tail transplant.

Care of Fishes

All fish were maintained in ordinary laboratory aquaria in the manner described by Gordon (1950). At the time of birth the members of a brood scheduled to serve as hosts were distributed over several aquaria in order to avoid overcrowding. They were fed live brine shrimp daily. Host fish thus treated withstood the operation very well, even if they received 2 fin grafts simultaneously; their mortality was below 1 per cent. Host fish that were raised under crowded conditions, however, had a postoperative mortality as high as 40 per cent. Such fish were usually stunted despite an adequate food supply, and their musculature was poorly developed; consequently, a pocket cut into their myotomes constituted a more serious wound than in fish with normal musculature. Grafts in young hosts hardly ever dislodged, whereas they were frequently lost from mature fish. After the operation, hosts were kept for 5 days in aquarium water to which was added 6 gm. sea salt per 1000 cc. as a precaution against bacterial and fungus infection. Unless otherwise stated, the water temperature averaged 25° C.

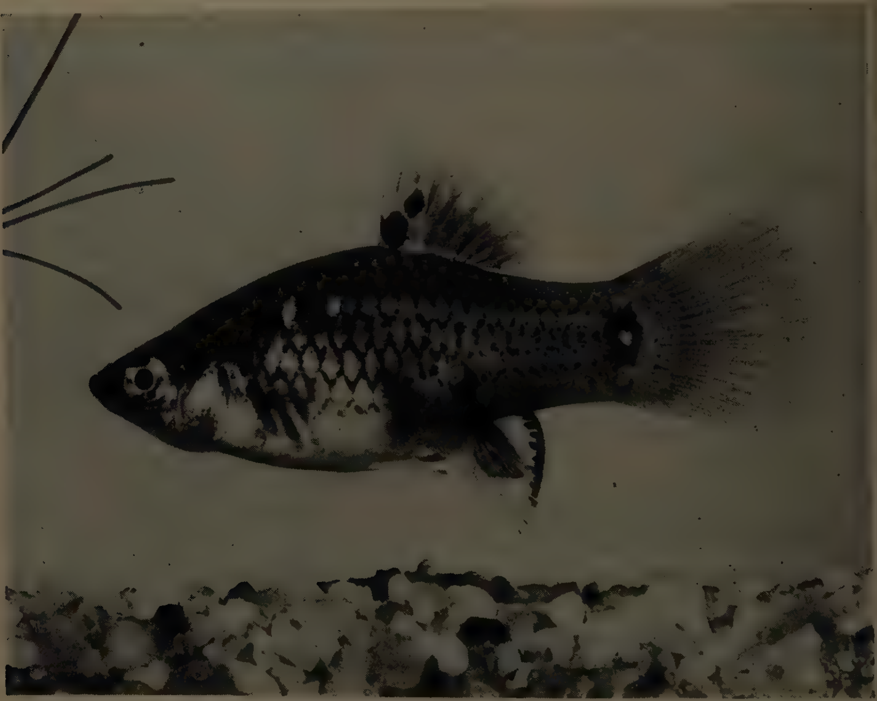

FIGURE 1. A female platyfish *Xiphophorus maculatus* of pedigree 859 (30 × 163) with an anal fin transplant from a donor of strain 163, photographed 18 months after the operation.

Criterion of Survival Time

A fin transplant was considered successful when the grafted fin survived as long as its host. In many cases these hosts were maintained for 2 years or more and used for further breeding experiments. In nearly all instances the transplanted fins developed and differentiated in a normal fashion. Unsuccessful fin transplants disintegrated without leaving any trace. The condition of a fin graft was judged by the appearance of its indigenous melanophores, guanophores, and lipophores. Chromatophores are arranged in characteristic patterns on the fin, and such pigment cells are normally absent elsewhere on the body, or they are distributed differently. For this reason it was possible to recognize even tiny remnants of grafted fin tissue. As long as any of the pigment cells of the graft were present it was considered alive. When all of its pigment cells had disintegrated, the graft was considered rejected. The validity of this criterion has been tested and confirmed repeatedly. The condition of homografts was checked daily for 3 weeks and thereafter twice weekly.

Definitions

A transplant from a donor all of whose histocompatibility genes are present in the host is known as an isotransplant. Transplants from donors whose histocompatibility genes are not present in the host are known as homotransplants. Transplants between hosts and donors belonging to 2 different species are known as heterotransplants.

For a description of the various phases through which iso- and homografts passed during the postoperative period, the following six terms are used:

Normal. A transplant that does not differ in its appearance from a fin in its normal position, except that it may have been distorted by operative injury and twisting.

Sloughing. The progressive disintegration of the distal margin of the transplant, while its proximal portion maintains a normal and healthy appearance. This sloughing process occurs both in isografts and homografts and appears to be due to the temporary denervation of the transplanted fin. A similar process has been described for the fins of *Fundulus* after temporary denervation of the pectoral fin (Goss and Stagg, 1957).

Only base left. A graft in which the sloughing process has stopped and only the base of the fin remains.

Regenerating. A fin transplant in which a regeneration blastema has formed over the distal edge of the fin stump and growth of a new fin has begun.

Disintegrating. A graft in which destructive changes other than the sloughing process occur throughout the fin. The disintegration is indicated by the destruction of pigment cells, fin rays, and interradial membrane.

Rejected. A graft in which all pigment cells have disintegrated.

RESULTS

Isografts

Kallman and Gordon (1958) reported that all intrastrain grafts (strain 30 to strain 30 and strain 163 to strain 163), all grafts from either inbred strain to F_1 hosts, and all grafts among F_1 hybrids were successful (FIGURE 2). These studies have now been extended to the F_2 and F_3 generations.

The anal fins of 28 randomly selected F_2 fish were transplanted into 28 F_1 hosts. All but one of these transplants were successful; the remaining one apparently failed to survive. This graft had been present on the fourth day after the operation, when these hosts were checked for the presence or absence of the grafts. A second check on day 18 failed to reveal any trace of it. This host was given a second transplant from another F_2 donor, and the new transplant survived.

Thirty anal fins from F_3 (850) donors were implanted into F_1 (859) hosts. Twelve of the donors were derived from a cross between 2 F_2 parents that rejected fins from a strain 30 as well as a strain 163 donor. Six donors were derived from F_2 parents that accepted fins from strain 30 and strain 163 donors.

Six were derived from a parent that accepted no graft and another that did accept a transplant from a strain 30 donor. Six were derived from a parent that accepted no graft and another that did accept a graft from a strain 163 donor.

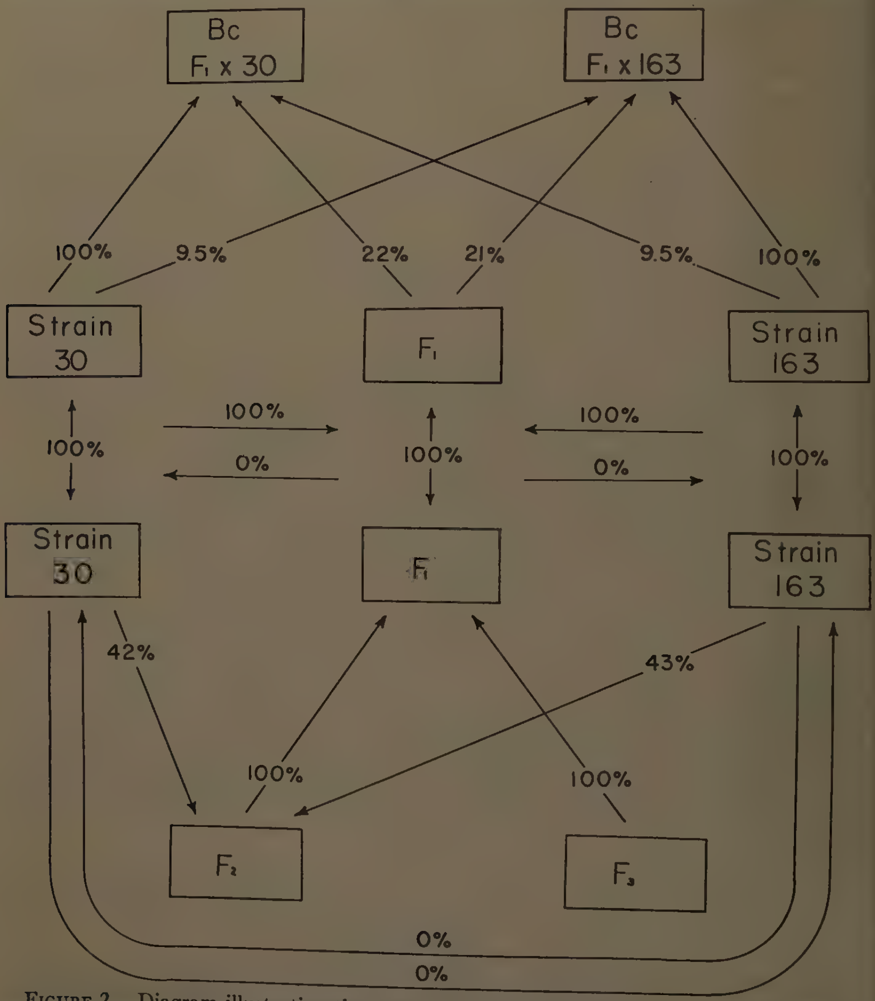

FIGURE 2. Diagram illustrating the genetics of fin transplantation in 2 inbred strains of platyfish, strains 30 and 163, and their F_1, F_2, F_3, and backcross (Bc) generations. The arrows point from the donors toward the hosts. Transplants are successful in 100 per cent of all cases from 30 to 30, 163 to 163, F_1 to F_1, 30 to F_1, 163 to F_1, F_2 to F_1, F_3 to F_1, 30 to Bc ($F_1 \times 30$), and 163 to Bc ($F_1 \times 163$). A certain percentage of transplants is successful from 30 to F_2, 163 to F_2, F_1 to Bc, 30 to Bc ($F_1 \times 163$), and 163 to Bc ($F_1 \times 30$). All transplants are rejected from 30 to 163, 163 to 30, F_1 to 30, and F_1 to 163.

All 30 transplants were successful, and their fate was indistinguishable from that of intrastrain grafts.

Homografts

All interstrain fin grafts (strain 30 into strain 163 and strain 163 into strain 30) were rejected between the fifth and fifteenth days. Transplants from F_1

hybrids to hosts of strains 30 and 163 survived considerably longer (Kallman and Gordon, 1958—*see* TABLES 1 and 2). Secondary interstrain grafts were rejected at an accelerated rate, and it made no difference whether the primary immunizing graft had come from a P_1 or F_1 donor (TABLE 2, FIGURES 3 and 4).

TABLE 1

SURVIVAL TIME OF F_1 AND P_1 GRAFTS BETWEEN 2 INBRED STRAINS OF PLATYFISH (30 AND 163)

Days after transplantation	30 to 163* (38 transplants)		F_1 to 163* (32 transplants)		163 to 30* (60 transplants)		F_1 to 30* (73 transplants)	
	Number	Per cent	Number	Per cent	Number	Per cent	Number	Per cent
3	—	—	—	—	—	—	—	—
4	—	—	—	—	—	—	—	—
5	1	2.6	—	—	—	—	—	—
6	3	7.8	—	—	6	10	—	—
7	11	29.0	—	—	10	16.6	—	—
8	19	50.0	—	—	15	25.0	2	2.7
9	24	63.1	—	—	23	38.3	—	—
10	28	73.6	2	6.2	33	55.5	—	—
11	33	86.8	2	6.2	47	78.3	8	10.9
12	34	89.4	3	9.3	55	91.6	—	—
13	37	97.3	—	—	59	98.3	18	24.6
14	37	97.3	6	18.7	60	100	—	—
15	38	100	—	—			28	38.2
16			8	25.0			—	—
18			12	37.5			45	61.6
20			19	59.3			54	73.9
22			22	68.7			57	78.0
24			23	71.8			62	84.9
28			27	84.3			64	87.6
33			27	84.3			66	90.4
42			29	90.6			68	93.1
57			31	96.8			73	100
67			32	100				

* Number and per cent of rejected transplants.

TABLE 2

SURVIVAL TIMES OF PRIMARY AND SECONDARY GRAFTS BETWEEN 2 INBRED STRAINS OF PLATYFISH AND THEIR F_1 HYBRIDS

Host strain	Primary donor	Median survival time*	Range (days)	Secondary donor†	Median survival time*	Range (days)
163	30	8	5 to 15	30	4	2 to 7
163	F_1	19	10 to 67	30	4	3 to 7
30	163	10	6 to 14	163	4	3 to 6
30	F_1	17	8 to 57	163	5	3 to 7
30	F_1	17	8 to 57	F_1	9	3 to 18

* Day on which 50 per cent of grafts were rejected.
† Implanted 28 days after primary graft.

Evaluation of survival time. It is established that no interstrain graft is successful. The validity of the criterion of considering a graft alive as long as at least some of its pigment cells were present and as rejected when all of its pigment cells had disintegrated was tested by transplanting fins from donors of strain 30 into hosts of strain 163 and retransplanting these grafts to a second strain 30 host a few days later. If this criterion is valid, then homografts that are already partially destroyed (classified as disintegrating) but in which some tissue still survives, as indicated by the presence of pigment cells, must survive after retransplantation to a genetically compatible host.

Sixty strain 163 hosts were given transplants from strain 30 donors. These hosts were divided into 12 groups of 5 fish each and kept in separate aquaria. On each of the following 12 days, the 5 grafts of 1 group were retransplanted into strain 30 hosts.

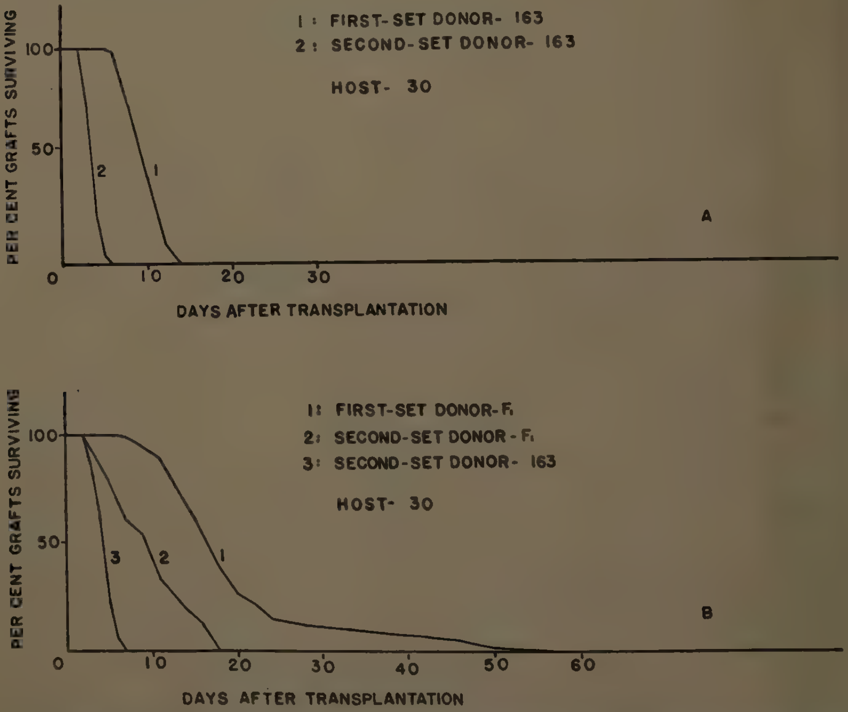

FIGURE 3. (*a*) A comparison of survival times of first-set strain 163 grafts with second-set strain 163 grafts in strain 30 hosts. The second-set grafts are rejected at an accelerated rate. (*b*) A comparison of survival times of first-set F_1 grafts with second-set F_1 and strain 163 grafts transplanted 28 days later into the same strain 30 hosts. The second-set F_1 grafts are rejected at an accelerated rate, but they survive longer than second-set P_1 grafts.

At the time of retransplantation 15 grafts were classified as normal, 5 as sloughing, and 11 as only base left. Twenty-nine of these 31 grafts survived permanently in their secondary hosts; 2 grafts, 1 normal, and the other only base left were lost due to mechanical difficulties. Their loss was in no way connected with any homograft reaction. Another 11 of the original 60 grafts were rated disintegrating, as indicated by the fragmentation of their fin rays and the degeneration of the pigment cells throughout the fin. Only 1 of these grafts failed to survive in its second host. Eighteen fin grafts were classified as rejected. One of these had apparently never undergone any sloughing at all, but all pigment cells had disappeared, and the graft, while still maintaining its normal shape and size, appeared pale and lifeless; after retransplantation, remnants of this graft could be found for 8 days in the strain 30 host. In 8 other hosts a few broken fin rays were all that was left of the graft; some of them fell apart during the process of retransplantation, and none of the other remnants persisted for more than 1 or 2 days. These results are summarized in TABLE 3.

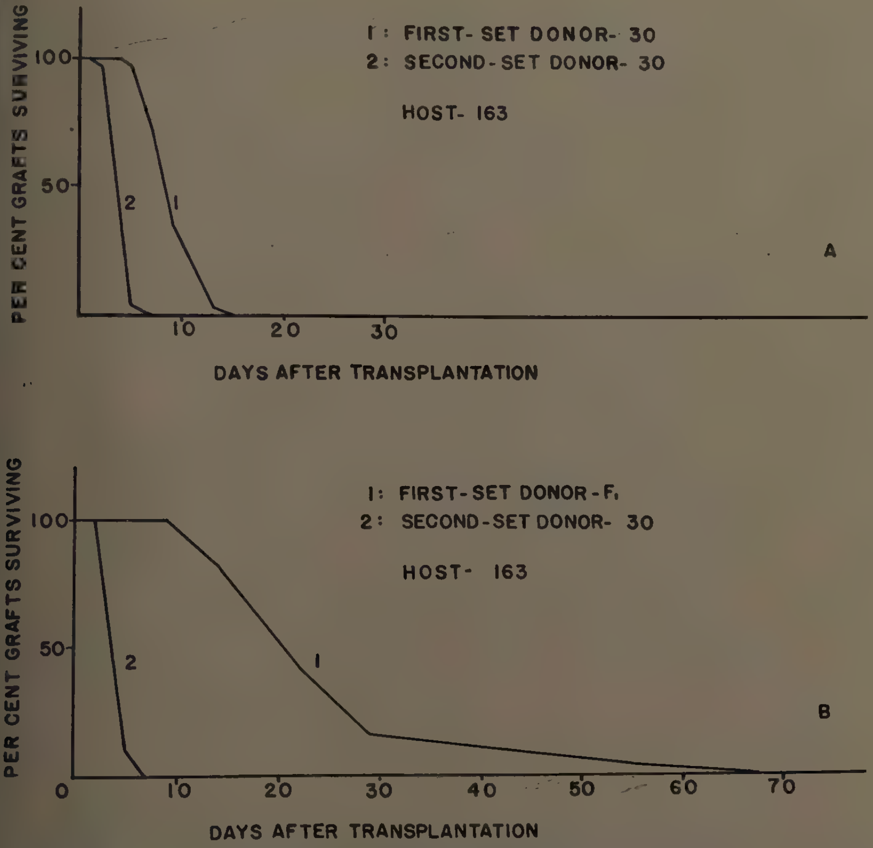

FIGURE 4. (*a*) A comparison of survival times of first-set strain 30 grafts with second-set strain 30 grafts in strain 163 hosts. The second-set grafts are rejected at an accelerated rate. (*b*) A comparison of survival times of first-set F_1 grafts with second-set strain 30 grafts transplanted 28 days later into the same strain 163 hosts. F_1 grafts survive longer than interstrain grafts.

Similar experiments were performed with secondary homografts (TABLE 4). Seven of the retransplanted secondary grafts were classified as normal and 11 as disintegrating; all 18 grafts survived retransplantation. In 12 other hosts a few broken fin rays were all that was left of each graft. They fell apart during the process of retransplantation.

As a control experiment, isotransplants (intrastrain grafts) were made among members of strain 30 and, after a few days, these grafts were retransplanted into a second strain 30 host. Since all hosts and donors belonged to the same inbred strain, genetic differences between them were negligible, and the failure of any graft to survive retransplantation must be attributed solely to mechanical difficulties. These grafts remained in their primary hosts from 7 to 12 days. At the time of retransplantation 2 were classified as normal, 2 as sloughing, and 1 as regenerating. All 5 survived in their second host. The remaining 25

TABLE 3

SURVIVAL OF PRIMARY HOMOGRAFTS AFTER RETRANSPLANTATION INTO GENETICALLY COMPATIBLE HOSTS

Condition* of 60 primary strain 30 grafts in strain 163 hosts					Number of days strain 30 grafts were in strain 163 hosts	Fate of strain 30 grafts after retransplantation into strain 30 hosts
N	S	B	D	R		
5†					1	All survived
5					2	4 survived, 1 lost accidentally
3	2				3	All survived
1	3	1			4	4 survived, 1 lost accidentally
		3	2		5	All survived
1		3		1	6	N and B grafts survived
		2	3		7	All survived
		2		3	8	B grafts survived
				5	9	None survived
			1	4	10	None survived
			3	2	11	D grafts survived
			2	3	12	D grafts survived

* N, normal; S, sloughing; B, only base left; D, degenerating; R, rejected.
† Number of grafts.

TABLE 4

SURVIVAL OF SECONDARY HOMOGRAFTS AFTER RETRANSPLANTATION INTO GENETICALLY COMPATIBLE HOSTS

Condition* of 30 secondary strain 163 grafts in strain 30 hosts					Number of days strain 163 grafts were in strain 30 hosts	Fate of strain 163 grafts after retransplantation into strain 163 hosts
N	S	B	D	R		
5†					1	All survived
2			3		2	All survived
			4	1	3	Four D grafts survived
			2	3	4	D grafts survived
			2	3	5	D grafts survived
				5	6	None survived

* N, Normal; S, sloughing; B, only base left; D, degenerating; R, rejected.
† Number of grafts.

grafts were classified as only base left. Two of them were lost during retransplantation because of technical difficulties. Of the remaining 23 grafts, only 1 failed to survive in its second host, in which it could be traced for 17 days before it was finally resorbed.

In most of the homo- and isografts that survived retransplantation, regeneration was at best abortive. Often only a few fin rays regenerated and, in some grafts, no regeneration took place; only the suspensorium (basal elements of the fin) survived permanently. In these cases, the integumentary tissue of the host had grown over the mouth of the pocket and prevented regeneration. However, when these fish were sectioned, the suspensorium of the grafts was found embedded in the musculature and appeared essentially normal. Those homo- or isografts that stayed in their primary hosts for only 5 days or less regenerated much better than grafts that had stayed in their first hosts for a longer period.

Secondary F_1 grafts. The difference in the number of foreign histocompatibility genes between F_1 and interstrain grafts is reflected in the longer survival of F_1 grafts, indicating a possible lower antigenicity for them. These F_1 grafts may survive longer because they are less efficient than P_1 grafts in inducing the host to produce a sufficient amount of antibody, or in the F_1 grafts there may be fewer avenues available over which the antibodies can attack. In order to test the latter possibility, fins were transplanted from F_1 donors into strain 30 hosts previously immunized by a primary F_1 graft. If the antibodies react with secondary F_1 grafts just as rapidly as with secondary P_1 grafts, then no difference between their survival times should be detected. Thirty strain 30 hosts in which the primary F_1 grafts had been rejected within the first 4 weeks after transplantation were given a secondary F_1 graft 28 days after the primary one. All these secondary grafts were rejected between day 3 and day 18, in contrast to secondary interstrain grafts, which were all destroyed by day 7 (TABLE 2 and FIGURE 3*b*). None of these secondary F_1 fin transplants showed any signs of regeneration. After the eleventh day, degenerative changes were visible in all fins that were still alive.

Simultaneous transplantation of strain 163 and F_1 grafts into strain 30 hosts. In a similar experiment a fin from a strain 163 donor and one from an F_1 hybrid were transplanted simultaneously into each of 28 hosts of strain 30. In each host the F_1 fin was implanted on the left side and the 163 fin on the right. Water temperature averaged 21° C. The condition of these grafts was checked every other day.

Fifty per cent of the strain 163 grafts had been rejected by the tenth day. Their survival times ranged from day 5 to day 18. Fifty per cent of the F_1 grafts had been rejected by day 19 (TABLE 5); their survival times ranged from day 10 to day 30.

With a single exception, all F_1 transplants survived from 4 days to 2 weeks longer than their corresponding 163 grafts (TABLE 6 and FIGURE 5). In 3 of the 5 hosts in which strain 163 grafts were still surviving on day 13, the stumps of the corresponding F_1 grafts showed indications of regeneration. On day 20, however, unmistakable signs of an incipient homograft reaction were present in all 3 hosts; all had ceased to grow, and some of their pigment cells had already disintegrated. These transplants survived until days 28 and 30. In the fourth host, the F_1 graft survived until day 22. The fifth host was exceptional in that both transplants disintegrated on day 15. Its strain 163 graft was one of the longer-surviving P_1 grafts, while its F_1 graft was one of the more short-lived ones. In 19 other hosts, the strain 163 grafts were rejected between days 7 and 13; their F_1 grafts, between days 18 and 25.

Both experiments show that the F_1 grafts are much more resistant to antibody attack than are interstrain grafts. Some F_1 grafts can survive for as long as 2 weeks in the presence of an antibody level sufficiently high to cause rapid rejection of interstrain grafts.

TABLE 5

STAGES IN THE BREAKDOWN OF F_1 AND STRAIN 163 GRAFTS TRANSPLANTED SIMULTANEOUSLY INTO 28 STRAIN 30 HOSTS

Condition of graft	Days after transplantation																							
	5		7		9		11		13		15		18		20		22		25		28		30	
	F_1	30	F_1	30	F_1	30	F_1	30	F_1	30	F_1	30	F_1	30	F_1	30	F_1	30	F_1	30	F_1	30	F_1	30
Normal	17	12	12	5	8	3	5		5		4		2		2									
Sloughing	7	6	5	4	4	1	1																	
Regenerating									3		4		4		1		1		1					
Only base left	4	3	11	6	13	5	15		13		7		4		4		2		1					
Degenerating		6		9	3	8	5	10	4	5	9	1	6		4		4		2		2			
Rejected		1		4		11	2	18	3	23	4	27	12	28	17	28	21	28	24	28	26	28	28	28

TABLE 6

SURVIVAL END POINTS OF STRAIN 163 AND F_1 GRAFTS TRANSPLANTED SIMULTANEOUSLY INTO 28 STRAIN 30 HOSTS

Number of hosts	Survival end points 163 grafts	(Days after transplantation) F_1 grafts
3	7	11 to 13
4	9	18 to 20
6	11	18 to 20
1	11	22
2	13	18
1	13	22
2	13	25
1	15	22
2	15	28 to 30
1	15	15
1	18	30
1	5	18
1	9	22
1	9	25
1	9	28

Determination of the Number of Histocompatibility Genes by Which Strains 30 and 163 Differ from Each Other

Transplants from P_1 donors into F_2 hosts. All transplants among members of an inbred strain (30 and 163) or from P_1, F_1, F_2, and F_3 donors to F_1 hosts (30 × 163) are successful. The genetic explanation of this assumes that no histocompatibility genes have been introduced, via the graft, that were not already present in the host. The members of the F_1 generation, therefore, are universal acceptors, since they share in single dose all the histocompatibility genes of either parent strain. Interstrain transplants are rejected, however, as are grafts from F_1 hybrids (30 × 163) to members of either parent strain. In such host-donor combinations histocompatibility genes not already present in the host are introduced via the graft.

TABLE 7 illustrates how 2 inbred strains (I and II) that differ at 2 histocompatibility loci and their F_1 and F_2 generations may be represented. Three of the classes shown (*AA BB*, *AA Bb*, *Aa BB*) possess the histocompatibility alleles *A* and *B* of strain I, but are lacking either all or one of the corresponding alleles of strain II. Therefore, in members of these 3 classes only transplants from donors of strain I survive. Conversely, 3 classes (*aa bb*, *Aa bb*, *aa Bb*) possess all the alleles of strain II, whereas they are lacking in one or all of those of strain I. In these hosts only transplants from donors of strain II are accepted. The complete heterozygotes are universal acceptors and accept grafts from either parent strain. The *AA bb* or *aa BB* hosts do not possess the full complement of histocompatibility genes of either parent strain, and in them transplants from donors of both strains are rejected. Therefore, the number of histocompatibility genes by which the members of 2 inbred strains differ from each other can be determined from the percentages of successful P_1 grafts in F_2 hosts. If 2 gene pairs are involved, 31.3 per cent of the F_2 members are expected to accept grafts from donors of 1 inbred strain, and another 31.3 per cent of the F_2 are expected to accept transplants from donors of the other inbred strain. Twenty-five per cent accept transplants from donors of both

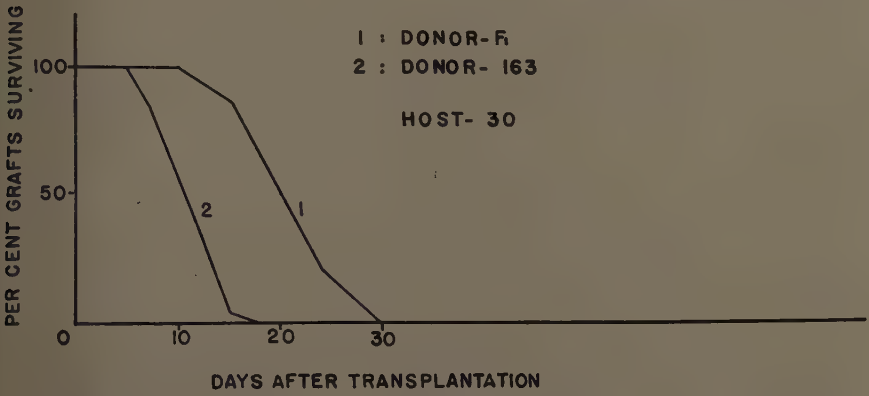

FIGURE 5. A comparison of survival times of F_1 and strain 163 grafts transplanted simultaneously into strain 30 hosts. The F_1 grafts survive considerably longer than the strain 163 grafts.

TABLE 7
SEGREGATION OF HISTOCOMPATIBILITY GENES WITH F_2 GENERATION

P_1 :	Strain I *AA BB* × Strain II *aa bb*			
F_1 :	*Aa Bb*			
	(Strain I acceptors)	(Universal acceptors)	(Strain II acceptors)	(No acceptors)
F_2 :	1 *AA BB* 2 *AA Bb* 2 *Aa BB*	4 *Aa Bb*	1 *aa bb* 2 *Aa bb* 2 *aa Bb*	1 *AA bb* 1 *aa BB*

strains, and in 12.5 per cent of the F_2 all transplants are rejected. The percentages when 1 to 5 loci are involved are listed in TABLE 8. This method of determining the number of histocompatibility genes assumes that the presence of a single foreign histocompatibility gene in the graft always leads to a homograft rejection. This method has been used to estimate the total number of histocompatibility loci involved in transplants of normal tissue between members of different strains of mice (Barnes and Krohn, 1957; Prehn and Main 1958).

From 8 F_1 (30 × 163) × F_1 (30 × 163) crosses (I to VIII) a total of 630 F_2 offspring was obtained (pedigree 793). Each of these fish received simultaneously 2 transplants, 1 from a donor of strain 30 and the other from a donor of strain 163. Seventy-one of these hosts (17.3 per cent) accepted both grafts, 156 (25.3 per cent) accepted only strain 30 grafts, 166 (26.8 per cent) accepted only strain 163 grafts, and in 188 (30.5 per cent) both grafts were rejected. Thirteen hosts died before the fate of one or both of their grafts could be ascertained (TABLE 9).

TABLE 8

PERCENTAGE OF TAKES AND REJECTIONS OF STRAIN 30 AND STRAIN 163 GRAFTS IN F_2 HOSTS

Number of genes involved	Expected percentage of hosts that will accept grafts from			
	Both strains	Strain 30 only	Strain 163 only	Neither strain
1	50	25	25	0
2	25	31.3	31.3	12.5
3	12.5	29.7	29.7	28.1
4	6.3	25.4	25.4	42.9
5	3.1	20.6	20.6	55.7
Experimental result:	17.3	25.3	26.8	30.5

	Total percentage of strain 30 takes	Total percentage of strain 163 takes
Expected for 2-factor ratio:	56.3	56.3
Expected for 3-factor ratio:	42.2	42.2
Expected for 4-factor ratio:	31.6	31.6
Experimental result:	42.6	44.2

These experimental data differ significantly from all expected theoretical ratios, but statistically they fit closest the expected ratio for 3 genes (TABLE 8). These results, therefore, indicate that strains 30 and 163 differ at at least 3, or possibly at 4, histocompatibility loci, and that under certain conditions transplants may survive although they possess some foreign histocompatibility genes.

Transplantation of F_1 and P_1 grafts into hosts of the backcross generations. The number of histocompatibility genes by which 2 inbred strains differ from each other may also be determined from the segregation of these genes in the backcross (Bc) generation:

F_1 × Strain I

Aa Bb *AA BB*

Bc: *AA BB Aa BB AA Bb Aa Bb*

Transplants from donors of strain II (*aa bb*) are expected to take only in the hosts of class *Aa Bb*. The other 3 classes lack 1 or both histocompatibility genes of strain II and, therefore, transplants from donors of strain II are not expected to survive in them. The expected percentages of takes when 1 to 5 genes are involved are listed in TABLE 10. Again, this method assumes that the presence of foreign histocompatibility genes in the graft always leads to homograft rejection.

TABLE 9

SUMMARY OF STRAIN 30 AND STRAIN 163 GRAFTS INTO F_2 HOSTS

Total number of F_2 hosts		Hosts with							
		Both grafts		Strain 30 grafts only		Strain 163 grafts only		No grafts	
		No.	Per cent	No.	Per cent	No.	Per cent	No.	Per cent
Series:									
793 I	157	29	18.7	47	30.3	38	24.5	41	26.1
II	109	19	17.4	30	27.5	28	25.7	29	26.6
III	165	29	17.7	36	22	53	32.3	46	28
IV	22	6	27.7	4	18.2	3	13.6	8	38.1
V	40	3	7.8	8	20.5	8	20.5	20	51.3
VI	66	10	15.6	14	21.9	16	25	24	37.5
VII	15	3	20	4	26.7	5	33.3	3	20
VIII	56	8	14.5	13	23.6	15	30.9	17	30.9
Total	617*	107	17.3	156	25.3	166	26.8	188	30.5

Total number of strain 30 takes: 263 (42.6 per cent).
Total number of strain 163 takes: 273 (44.2 per cent).
* Thirteen fish died after day 28 and day 56.

TABLE 10

PERCENTAGE OF P_1 AND F_1 TAKES IN BC HOSTS

Number of genes involved	Expected percentage of	
	P_1 takes	F_1 takes
1	50	50
2	25	25
3	12.5	12.5
4	6.3	6.3
5	3.1	3.1

Experimental results	P_1 takes	F_1 takes
Series 816 I (strain 30 and F_1 grafts)*	11	15.7
Series 847 I (strain 163 and F_1 grafts)*	9	15.0
Series 816 II (F_1 grafts only)		21.3
Series 847 II (F_1 grafts only)		22.0
Series 816 III (strain 30 grafts only)	9.4	
Series 847 III (strain 163 grafts only)	9.7	
Total percentage of P_1 takes	10.0%	

* Both grafts implanted in the same host.

Seventy-two hosts (pedigree 847 III) of the Bc generation F_1 (30 × 163) × 30 received transplants from donors of strain 163. Seven (9.8 per cent) of these were successful, while all others were rejected (TABLE 11 and FIGURE 6*a*). In the reciprocal experiment 139 fish (pedigree 816 III) of the Bc generation F_1 (30 × 163) × 163 received transplants from donors belonging to strain 30. Thirteen (9.4 per cent) of these were successful, while all others were rejected (TABLE 12 and FIGURE 7*a*).

These experimental data again indicate a segregation of 3, possibly 4, histocompatibility loci. These data do not differ significantly from the theoretical expectation of 12.5 per cent for 3 loci or 6.3 per cent for 4 loci.

TABLE 11

SUMMARY OF STRAIN 163 AND F_1 TRANSPLANTS INTO Bc 847 (F_1 × 30) HOSTS

Days after transplantation	Number of rejected grafts							
	Bc 847 I hosts (total 98)*				Bc 847 II hosts (total 59)**		Bc 847 III hosts (total 72)†	
	Strain 163 grafts	Per cent	F_1 grafts	Per cent	F_1 grafts	Per cent	Strain 163 grafts	Per cent
7	8	8.2	0	0	0	0	8	11.1
15	37	37.4	20	20.4	10	16.9	33	45.8
22	63	64.3	44	44.9	24	40.7	45	62.9
29	76	77.5	67	68.4	34	57.6	53	73.4
36	82	83.7	71	72.4	37	62.7	58	80.0
43	82	83.7	77	78.6	40	67.8	60	83.3
57	85	86.7	78	79.6	44‡	74.6	62	86.1
64	87	88.8	80	81.6	44	74.6	63	87.5
82	87	88.8	80	81.6	44	74.6	63	87.5
94	88	89.8	81	82.6	45	76.3	64	88.9
110	88	89.8	82	83.7	45	76.3	65	90.2
124	88	89.8	82	83.7	45	76.3	65	90.2
140§	89	90.8	83	84.7	46	78.0	65	90.2
Number of takes	9	9.2	15	15.3	13	22	7	9.8

* Each host received simultaneously 2 transplants, 1 from an F_1 donor and 1 from a strain 163 donor.

** Each host received a single F_1 graft.

† Each host received a single strain 163 graft.

‡ Two hosts died with their F_1 grafts still in good condition.

§ After day 140 there were no more changes.

The lack of close agreement between the theoretical and actual percentages suggests that, under certain conditions, the presence of foreign histocompatibility genes in the cells of the graft and their absence from the cells of the host may not always lead to a destruction of the transplanted tissue. This possibility was critically tested in the following experiment. If a difference of 2 genes is assumed, the Bc generation falls into 4 classes, as shown above.

Neither an *aa bb* donor nor an F_1 donor (*Aa Bb*) introduces foreign histocompatibility genes into a Bc host with an *Aa Bb* genotype. An interstrain transplant from strain II (*aa bb*) differs from a host of the genotype *Aa BB* by 2 *b* genes, from an *AA Bb* host by 2 *a* genes, and from an *AA BB* host by 4 genes (2 *a* and 2 *b* genes). However, an F_1 donor differs from an *AA Bb* or

Aa BB host by only a single histocompatibility gene. If, therefore, under certain conditions transplants can survive in spite of the presence of foreign histocompatibility genes, then more F_1 than P_1 transplants should survive in the Bc hosts. If, however, the presence of a foreign histocompatibility gene always leads to a homograft reaction, then the percentages of successful F_1 and P_1 grafts in the hosts of the Bc generation must be the same!

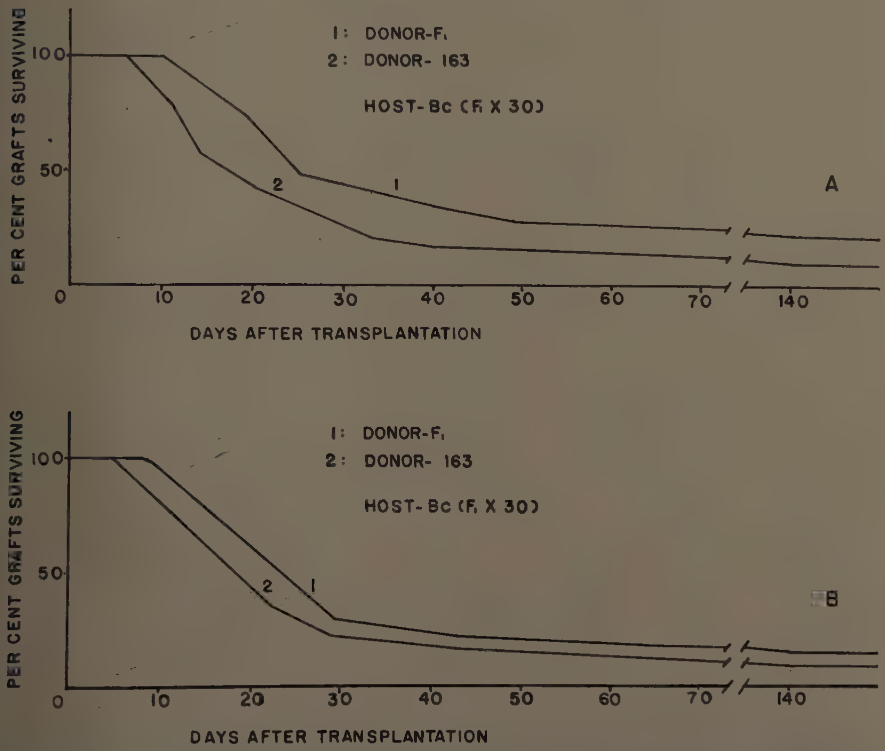

FIGURE 6. (*a*) A comparison of survival times of F_1 and strain 163 grafts transplanted into different Bc ($F_1 \times 30$) hosts. The percentage of F_1 takes is higher than that of successful P_1 takes, and the rate of rejection of F_1 grafts is slower. (*b*) A comparison of survival times of F_1 and strain 163 grafts transplanted simultaneously into the same Bc ($F_1 \times 30$) hosts. The percentage of successful F_1 grafts is slightly higher than that of P_1 grafts, and the F_1 grafts survive slightly longer. However, the differences between the F_1 and strain 163 grafts are less pronounced than those in *a*.

Fifty-nine hosts (pedigree 847 II) of the Bc F_1 (30 × 163) × 30 received transplants from F_1 hosts. Thirteen (22 per cent) of these were successful (TABLE 11 and FIGURE 6*a*). In the reciprocal experiment 89 hosts (pedigree 816 II) of the Bc generation of F_1 (30 × 163) × 163 received transplants from F_1 donors. Nineteen (21.3 per cent) of these transplants were successful (TABLE 12, FIGURE 7*a*). The percentage of successful F_1 grafts in Bc hosts is significantly higher than the percentage of P_1 takes. These experiments demonstrate clearly that some transplants containing 1 or possibly more histocompatibility genes that are absent from the hosts can survive.

It is therefore of great interest to determine what happens if a Bc host receives fin transplants simultaneously from a P_1 donor and an F_1 donor. In such an experiment both the P_1 and F_1 grafts differ from their Bc host by the same histocompatibility gene(s), but the P_1 donor differs from the host by twice as many genes as the F_1 donor. Ninety-eight hosts (pedigree 847 I) of the Bc F_1 (30 × 163) × 30 each received simultaneously 2 transplants, one from a strain 163 and the other from an F_1 donor. In 9 fish (9.2 per cent) both grafts were successful. In 6 (5.8 per cent) only the F_1 graft survived, and in all other hosts both grafts were rejected (TABLE 11, FIGURE 6*b*). In the reciprocal experiment 127 Bc hosts (pedigree 816 I) of the cross F_1 (30 × 163) × 163 each received simultaneously 2 transplants, one from a strain 30 donor and the other from an F_1 donor. In 14 fish (11 per cent) both grafts were successful, in 6 fish (4.7 per cent) only the F_1 fins survived, and in the remaining hosts both grafts were rejected (TABLE 11, FIGURE 7*b*). Among these 225 Bc hosts that received both F_1 and P_1 grafts, there was not a single host in which only the F_1 transplant was rejected while the P_1 graft survived.

TABLE 12

SUMMARY OF STRAIN 30 AND F_1 TRANSPLANTS INTO BC 816 (F_1 × 163) HOSTS

Days after transplantation	Number of rejected grafts							
	Bc 816 I hosts (total 127)*				Bc 816 II hosts (total 89)**		Bc 816 III hosts (total 139)†	
	Strain 30 grafts	Per cent	F_1 grafts	Per cent	F_1 grafts	Per cent	Strain 30 grafts	Per cent
7	4	3	0	0	0	0	—	—
10	22	17.3	6	4.7	1	1.1	28	20.1
12	42	33.1	20	15.7	4	4.5	39	28.1
17	79	62.2	43	33.8	13	14.6	65	46.8
21	87	68.5	55	43.3	24	27.0	90	65.7
32	98	77.1	80	63.0	36	40.4	108	77.7
41	100	78.7	88	69.4	43	48.3	114	82.0
54	104	81.8	94	74.0	53	59.5	115	82.8
68	108	85.0	98	77.2	57	64.1	120	86.3
85	110	86.6	102	80.3	61	68.5	124	89.2
94	110	86.6	102	80.3	63	70.7	125	89.9
110	111	87.4	104	81.8	67	75.3	126	90.6
120	113	89.0	106	83.4	68	76.4	126	90.6
145‡	113	89.0	107§	84.3	70	78.7	126	90.6
Number of takes	14	11.0	20	15.7	19	21.3	13	9.4

* Each host received simultaneously two transplants, one from an F_1 donor and one from a strain 30 donor.

** Each host received a single F_1 graft.

† Each host received a single strain 30 graft.

‡ After day 145 there were no more changes.

§ One host died with its F_1 graft still present.

The 6 Bc 816 I hosts in which only the F_1 grafts survived were challenged on day 120 with a second transplant from a strain 30 donor. Two of these fish died accidentally after the operation. In the remaining 4 the secondary strain 30 transplants were rejected within 10 days, while there was no change in the healthy appearance of their F₁ grafts. These experiments, therefore, provide conclusive evidence that under certain conditions some transplants can survive not only when they possess at least 1 foreign histocompatibility gene, but also in the presence of host antibodies against its corresponding antigen.

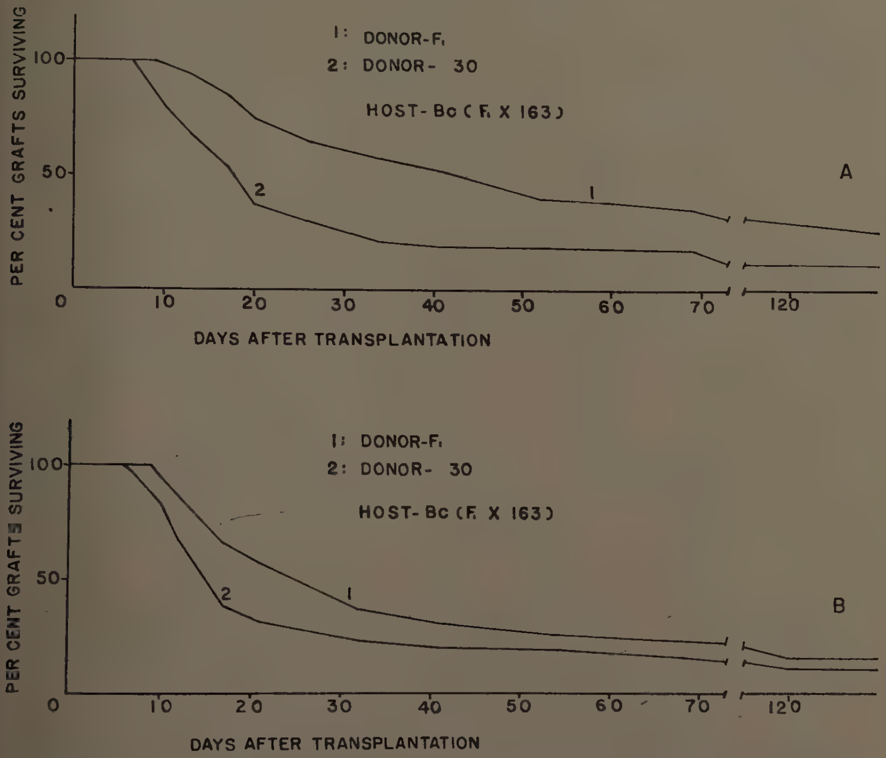

FIGURE 7. (*a*) A comparison of survival times of F₁ and strain 30 grafts implanted into different Bc (F₁ × 163) hosts. The percentage of successful F₁ grafts is higher than that of P₁ grafts, and the rate of rejection of F₁ grafts is slower. (*b*) A comparison of survival times of F₁ and strain 30 grafts transplanted simultaneously into the same Bc (F₁ × 163) hosts. The percentage of successful F₁ transplants is slightly higher than that of P₁ grafts, and the F₁ grafts survive slightly longer. However, the differences between the F₁ and strain 30 grafts are less pronounced than those in *a*.

Transplantation of Fins from Strain 163 *Donors to Bc* (*F*₁ × 163) *Hosts and from Strain* 30 *Donors to Bc* (*F*₁ × 30) *Hosts*

As a control experiment 15 Bc (F₁ × 163) hosts received fins from strain 163 donors, and 15 Bc (F₁ × 30) hosts received fins from strain 30 hosts. All transplants were successful.

Survival Times of P₁ Grafts in F₂ Hosts

Whereas interstrain grafts survived only 1 or 2 weeks, P₁ grafts in F₂ hosts survived from 10 to 180 days (TABLE 13). The condition of these grafts was checked and recorded on days 4, 14, 28, 56, 80, 120, 150, 180, and 360. After 80 days degenerative changes could be observed in all the grafts that were eventually rejected. In all transplants surviving at least 80 days the homograft reaction proceeded extremely slowly and extended over several weeks or months. In such transplants the first signs of an incipient homograft reaction were a general retardation of growth or lack of regeneration following the sloughing process, followed later by a gradual disintegration of pigment cells. Many P_1 donors differ from their F_2 hosts only by 2 histocompatibility genes, and grafts from these may therefore survive for long periods of time.

TABLE 13

SURVIVAL TIMES OF STRAIN 30 AND STRAIN 163 GRAFTS IN 630 F_2 (793) HOSTS*

Days after transplantation	Number of hosts carrying			
	Both grafts	Strain 30 grafts only	Strain 163 grafts only	No grafts
14	297	140	160	33
28	207	164	179	80
56	146	167	194	120
80	128	163	181	145
120	114	159	174	170
150	107	157	170	183
180	107	156	166	188

* Thirteen hosts died after days 28 and 56.

TABLE 14

THE FATE OF 139 STRAIN 30 GRAFTS TRANSPLANTED SINGLY INTO BC 816 III HOSTS

Condition of graft	Days after transplantation														
	10	13	17	20	26	34	41	52	68	80	92	103	112	120	... 250
Normal	5	1	1	1	1	1	16	14	13	13	13	13	13	13	13
Regenerating		11	20	21	20	17									
Only base left	73	47	30	12	10	7	4	4	3	1					
Degenerating	33	35	23	17	10	3	5	7	3	2	1				
Rejected	28	45	65	88	98	111	114	114	120	123	125	126	126	126	126

Survival Times of F_1 and P_1 Transplants Grafted Singly into Backcross Hosts

The survival times of 191 unsuccessful P_1 grafts in Bc III hosts ranged from day 7 to day 120 (TABLES 14 and 15, FIGURES 6*a* and 7*a*). Some regeneration occurred in at least 11 grafts before they were rejected. In 2 of them growth soon ceased (around day 26), and they were rejected within the next 2 weeks. In 4 other grafts the first indications of incompatibility were noticed at about day 34, but in these the homograft reaction proceeded more slowly; they survived until days 47, 54, and 62 (2 grafts), respectively. In a seventh host the growth of the transplant ceased around day 41, rejection proceeded slowly, and it survived until day 80. In the eighth host the transplant ceased to grow at about day 47, when the first disintegration of pigment cells was also noticed; the condition of the graft deteriorated rather slowly, and it survived until day

115. Two other grafts began to break down at about day 52; one was rejected rather rapidly and had disintegrated completely by day 68, while the other graft survived until day 80. In the eleventh host the homograft reaction began at about day 68; parts of this transplant survived until day 103.

In 2 hosts the grafts did not undergo any sloughing after implantation. They maintained their normal appearance until days 34 and 54, respectively. Rejection proceeded extremely slowly; the first graft survived until day 68, the other until day 94. Six grafts that never underwent regeneration nevertheless survived for more than 2 months. One of these was rejected on day 61, 2 on day 68, 1 on day 80, and 2 on day 94.

TABLE 15

THE FATE OF 72 STRAIN 163 GRAFTS TRANSPLANTED SINGLY INTO Bc 847 III HOSTS

Condition of graft	Days after transplantation														
	7	11	14	19	25	33	40	47	54	61	84	94	108	120	... 250
Normal	8	5	4	4	4	4	9	8	7	7	7	7	7	7	7
Regenerating			3	7	7	7									
Only base left	37	26	14	9	3										
Degenerating	23	26	20	10	10	4	3	3	3	2	2	1	1		
Rejected	4	15	31	42	48	57	60	61	62	63	63	64	64	65	65

TABLE 16

THE FATE OF 89 F_1 GRAFTS TRANSPLANTED SINGLY INTO Bc 816 II HOSTS

Condition of graft	Days after transplantation																
	10	13	17	20	26	34	41	52	59	68	81	94	110	120	132	145	... 250
Normal	6	4	4	3	3	1	24	22	21	21	19	19	19	19	19	19	19
Regenerating		14	29	30	29	28											
Only base left	76	53	32	22	19	17	12	6	6	2	1						
Degenerating	6	13	11	12	5	6	10	8	8	9	9	7	3	2	2		
Rejected	1	5	13	22	33	37	43	53	54	57	60	63	67	68	68	70	70

The survival times of the 114 unsuccessful F_1 grafts in the Bc 816 II and Bc 847 II hosts also ranged from day 10 to day 145 (TABLES 16 and 17; FIGURES 6*a* and 7*a*). Although the range of their survival times was very similar to that of the P_1 grafts in Bc hosts, their average survival time was considerably longer. Fifty per cent of the unsuccessful P_1 grafts had been rejected by day 17, while 50 per cent of the unsuccessful F_1 grafts survived until at least day 28. The 75 per cent mark was reached by the P_1 grafts at about day 25, and by the F_1 grafts at about day 45. Apparently, F_1 grafts survive longer because they possess only one half as many foreign histocompabibility genes as do P_1 grafts.

As in many previous transplants the homograft reaction proceeded slowly. Thirteen of the unsuccessful grafts underwent regeneration before the first sign of the homograft reaction was noticed. In one of these grafts the first degenerative changes were observed as early as day 26, and the transplant survived only until day 41. A second transplant ceased to grow at about day 34, but its rejection was extremely slow; it survived until day 94. Four other regenerating transplants ceased to grow at about day 40, underwent slow degeneration, and survived until days 94, 110 (2 grafts), and 145, respectively. Three other regenerating grafts began to disintegrate at about days 47 to 52 and survived until days 81 and 94 (2 grafts), respectively. In 2 other grafts the first disintegration of pigment cells was observed at about day 59; the homograft reaction then proceeded slowly, and these grafts were not rejected until day 140. Finally, in 2 hosts the growth of these transplants continued until day 68, followed by a gradual disintegration; these grafts survived until days 110 and 120, respectively.

The transplants in 3 hosts never underwent the sloughing process after implantation. In 2 of them the first degenerative changes were observed on day 34, and they had completely disintegrated by days 41 and 52. The graft of the third host began to break down on day 41 and was rejected on day 81.

TABLE 17

THE FATE OF 59 F_1 TRANSPLANTS GRAFTED SINGLY INTO BC 847 II HOSTS

Condition of graft	Days after transplantation														
	7	11	14	19	25	33	40	47	54	61	84	94	125	140	... 250
Normal	21	7	4	1			17	14*	14	13	13	13	13	13	13
Regenerating			14	17	17	17									
Only base left	35	40	16	16	3	2									
Degenerating	3	9	17	9	8	4	3	2	1	2	2	1	1		
Rejected		3	8	16	31	36	39	41	42	42	42	43	43	44	44

* Two hosts died with grafts still in excellent condition.

Two transplants never underwent regeneration, but their proximal portions survived for prolonged periods of time. One was rejected on day 81 and the other on day 110.

Survival Times of P_1 and F_1 Transplants Grafted Simultaneously into Bc Hosts

If the Bc host possesses most of the histocompatibility genes of the P_1 donor, the P_1 graft would be expected to survive for a considerable period of time and the F_1 graft as long or longer. In about 85 per cent of all cases the survival times of both grafts differed by less than 4 weeks. The fate of each graft pair is described in TABLES 18 and 19. In the second columns of these tables the microscopic appearance of the grafts as they appeared on the seventeenth postoperative day is listed; before that time the changes occurring within the grafts were too numerous and too rapid for following up the history of individual transplants. The day on which the first disintegration of pigment cells was observed is listed in the third column. In many transplants the incipient homograft reaction was already indicated as early as day 17 by the absence of any regeneration, although the actual disintegration of tissue may not have been observed for several days or weeks.

However, in only 10 hosts (11 per cent) of the Bc 847 I series did the fates of the strain 163 and F₁ grafts differ strongly (TABLE 18). For example, in 4 of these the F₁ grafts survived from 1 to 2½ months longer than their corresponding strain 163 grafts. In the other 6 cases the strain 163 grafts were rejected, while their F₁ transplants survived indefinitely. Nevertheless, in only 8 of 83 hosts in which both grafts were rejected did the strain 163 grafts survive for more than 30 days. In only 1 of the 6 hosts in which only the F₁ grafts survived permanently was the strain 163 graft rejected before day 30. This indicates that the factors that enabled the F₁ grafts to survive permanently also had an influence, although a somewhat smaller one, upon the survival times of the P₁ strain 163 grafts.

TABLE 18

THE FATES OF STRAIN 163 AND F₁ TRANSPLANTS GRAFTED SIMULTANEOUSLY INTO 98 Bc 847 (F₁ × 30) HOSTS

Number of hosts	Condition of grafts on day 17*		First sign of homograft reaction (days after transplantation)		Survival end point of grafts (days after transplantation)	
	163	F₁	163	F₁	163	F₁
22	—	—	—	—	17‡	17‡
10	—	D	—	15*	17‡	22–24
3	—	B	—	—	17‡	29
1	—	B	—	—	17‡	36
1	—	B	—	—	17‡	39
1	—	N	—	22	17‡	29
12	D	D	15‡	—	22	22
11	D	B	15†	20	22	29
1	D	B	15†	20	24	33
1	D	B	15	25	22	36
1	D	B	15	36	29	43
7	D	B	15	22	29	29
1	D	B	17	33	33	53
1	B	B	22	22	29	29
2	B	B	22	36	36	43
1	B	B	20	29	29	43
1	B	B	20	36	29	54
1	B	R	24	33	33	53
1	B	B	29	29	54	63
2	B	B	—	—	permanent survival	
7	R	R	—	—	permanent survival	
1	D	R	15	43	29	64
1	B	R	36	36	57	94
1	B	R	28	54	39	110
1	N	R	57	64	64	140
1	—	R	—	—	17‡	permanent survival
1	B	R	25	—	36	permanent survival
1	R	R	33	—	39	permanent survival
1	R	R	36	—	60	permanent survival
1	R	R	36	—	94	permanent survival
1	R	R	43	—	135	permanent survival

* Key: D, degenerating; B, only base left; N, sloughing; R, regenerating.
† On or before day 15.
‡ Rejected on or before day 17.

In 16 hosts (14 per cent) of the Bc 816 I series there was a considerable difference between the survival times of the F_1 and the strain 30 transplants (TABLE 19). In 10 of these the F_1 grafts survived 3 to 5 times as long as their corresponding strain 30 grafts, and in only 6 the P_1 grafts were rejected. Significantly, in these 6 hosts the strain 30 grafts survived for a period of 2 to 3 months, thus indicating, once more, that the factors allowing the F_1 grafts to survive permanently also contributed to a prolonged survival of the P_1 grafts.

TABLE 19

THE FATE OF STRAIN 30 AND F_1 TRANSPLANTS GRAFTED SIMULTANEOUSLY INTO 127 Bc 816 (F_1 × 163) HOSTS

Number of hosts	Condition of grafts on day 17*		First sign of cell disintegration (days after transplantation)		Survival end point of grafts (days after transplantation)	
	30	F_1	30	F_1	30	F_1
42	—	—	—	—	17†	17†
5	—	D	—	17†	17†	21
12	—	D	—	17†	17†	26
3	—	D	—	17†	17†	32
5	—	B	—	20 to 25	17†	32
3	—	B	—	26	17†	41
2	D	B	17†	20	21	26
3	D	D	17†	17†	26	26
1	D	B	17†	20	26	26
2	D	D	17†	17	32	32
2	B	B	?	20	21	26
1	B	R	20	26	26	32
1	B	B	20	26	26	41
1	B	B	20	26	32	32
1	B	B	26	26	32	41
1	B	R	21	32	32	41
2	B	B	26	32	32	41
1	B	B	32	32	41	54
1	B	B	40	48	54	68
1	B	B	40	54	68	85
1	B	B	41	85	68	110
1	B	B	54	54	120	120
1	N	R	32	41	41	68
1	N	B	54	54	85	85
1	R	R	41	68	110	110
1	R	R	68	68	120	120
14	R	R	—	—	permanent survival	
2	—	B	—	32	17†	54
3	—	B	—	41	17†	54
1	—	B	—	41	17†	68
1	—	B	—	54	17†	85
1	—	N	—	32	17†	54
1	—	N	—	54	17†	85
1	D	B	17†	85	21	110‡
1	B	N	41	—	54	permanent survival
1	B	R	41	—	54	permanent survival
1	R	N	32	—	54	permanent survival
2	B	R	41	—	68	permanent survival
1	B	R	68	—	85	permanent survival
1	D	—	17	—	21	17†

* Key: D, degenerating; B, only base left; R, regenerating; N, normal.
† On or before day 17.
‡ Host died on day 110 with the proximal portion of the F_1 graft still intact.

In contrast, in only 10 of the 107 hosts in which both grafts were rejected did the strain 30 grafts survive for more than 30 days.

DISCUSSION

Perhaps the best demonstration that ordinarily transplants can survive only in hosts into which they do not introduce any foreign histocompatibility alleles is provided by the successful transplantation of all fins from F_2 and F_3 donors into F_1 hosts. The members of the F_2 and F_3 generations are extremely heterozygous, and the existence of 24 pairs of chromosomes in this species makes it unlikely that any 2 F_2 or F_3 fish will be genetically alike or will have the same genotype as the F_1 hosts. Nevertheless, none of the F_2 and F_3 donors can possess any allele that is not represented in the F_1 hybrids. Therefore, F_2 and F_3 fins should survive when transplanted into F_1 hosts, and this expectation is realized in the present experiment.

Actually, in these experiments the F_2 and F_3 donors were not the direct descendants of the F_1 generation used as hosts here ($30^{24} \times 163^{12}$). Instead, they had descended from the F_1 generation that had been obtained from the cross $30^{23} \times 163^{11}$. Therefore, both host and donor had descended from a common 30^{22} and 163^{10} ancestor, but several generations separated them. That all but 1 of these transplants were successful indicates that, with the 1 possible exception, no significant change at any of the histocompatibility loci had occurred during the course of these generations.

Number of Histocompatibility Genes

A calculation of the number of histocompatibility loci that control the fate of transplants between members of 2 inbred strains depends upon their Mendelian segregation in the F_2 and Bc generations. Thus the number of individuals in the F_2 and Bc generations that possess at least a single dose of the histocompatibility genes of either of the parental strains is directly related to the total number of histocompatibility genes involved. Such F_2 and Bc individuals will accept grafts from P_1 donors. The theoretical percentages of successful transplants when from 1 to 5 histocompatibility genes are involved are shown in TABLES 8 and 10. This method assumes that a difference at a single histocompatibility locus will always lead to a rejection of the graft.

The results obtained from transplanting strain 30 and strain 163 fins into F_2 hosts (TABLE 8) best fit a 3-factor ratio, indicating that at least 3 histocompatibility loci must be involved. There are no significant differences between the actual and theoretical percentages for the F_2 groups that accepted strain 30 grafts only, strain 163 grafts only, and no grafts. The percentage of F_2 fish that accepted both grafts was significantly higher than theoretical expectation. Despite this, the total percentages of successful strain 30 grafts and of successful strain 163 grafts are nevertheless in close agreement with the theoretical expectation for 3 factors.

The results obtained from grafting strain 30 and strain 163 fins into hosts of the Bc generations also support the tentative conclusion that at least 3 different histocompatibility genes must be involved (TABLE 10). The percentage of successful P_1* grafts was almost 10 per cent (43 of 436), which again is in fairly close agreement with the theoretical expectation of 12.5 per cent for 3 factors. This difference of 2.5 per cent between the actual result and theoretical expectation is not statistically significant.

Since the F_1 donors possessed the same histocompatibility alleles as P_1 donors (although the latter possessed them in double dose), the percentages of successful P_1 and F_1 transplants in Bc hosts should have been the same. In series Bc 816 II and Bc 847 II, in which each host received a single F_1 transplant, however, the percentage of successful F_1 grafts was approximately 22 per cent, more than twice as high as the percentage of P_1 takes in series Bc 816 I and III and Bc 847 I and III. These differences are significant. On the other hand, when F_1 and P_1 grafts were transplanted simultaneously into Bc hosts the percentage of accepted F_1 grafts dropped to about 15 per cent, which, however, is still higher than the percentage of successful P_1 transplants (TABLE 10). Moreover, the very fact that, in both series Bc 816 I and Bc 847 I, 12 of 225 hosts accepted F_1 grafts while rejecting P_1 grafts clearly indicates that some F_1 grafts can survive even though they introduce at least 1 foreign histocompatibility allele. That the rejection of these P_1 grafts was not due to experimental error is shown by the fact that, when a secondary P_1 graft was transplanted into some of these hosts, a typical second-set response was obtained against the P_1 graft, while the F_1 graft remained unchanged.

Additive and Dosage Effects

In contrast to the interstrain grafts, where host and donor differ at the maximum possible number of histocompatibility loci, many P_1 grafts differ from their F_2 and Bc hosts at only 1 or 2 loci, and the grafts of these host-donor combinations are undoubtedly the ones that survive for long periods of time. The rapid rejection of interstrain grafts, therefore, appears to be due to the additive effects of a number of histocompatibility genes.

Theoretically, 14.3 per cent of all Bc hosts that reject their grafts differ from the P_1 donors at 3 histocompatibility loci, 42.9 per cent at 2 loci, and 42.9 per cent at 1 locus. In contrast, only 2.7 per cent of the hosts of the F_2 generation that reject their P_1 grafts differ from the P_1 donors at 3 histocompatibility loci, 24.3 per cent at 2 loci, and 73 per cent at a single locus. Since the percentage of hosts that differ from their P_1 donors at a single locus is much higher among the F_2 than among the Bc, the rate of graft rejection should have been much slower among the F_2 than among the Bc hosts if histocompatibility genes have an additive effect.

There was no difference in the survival times of the first 50 per cent of rejected grafts between the F_2 and Bc hosts, but the 75 per cent mark was reached slightly later in the F_2 hosts, and the 87.5 per cent mark was reached 24 to 38 days later than in the Bc hosts (TABLE 20).

A comparison of the survival times of F_1 and P_1 grafts shows that generally F_1 grafts survived significantly longer than P_1 grafts. These results mean that each histocompatibility allele produces a limited quantity of specific antigen, and that if an allele is present in a homozygous condition about twice as much specific antigen is produced as in a heterozygous graft where each allele is represented only once. The antigenicity of a graft is thus the sum of the dosage and additive effects of the histocompatibility alleles in question. The speed of homograft rejection, in turn, depends upon the antigenicity of the graft.

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* In this discussion any reference to P_1 grafts and their Bc hosts refers to the following 2 host-donor combinations: P_1 30 into Bc 816 (F_1 × 163) and P_1 163 into Bc 847 (F_1 × 30). The 2 other possible combinations, P_1 30 into Bc 847 and P_1 163 into Bc 816, are not considered in this connection.

While the demonstration of a dosage effect is relatively clear-cut in the present experiments, attempts to demonstrate dosage effects for histocompatibility genes in the house mouse have proved inconclusive (Billingham *et al.*, 1954; Barnes and Krohn, 1957; Prehn and Main, 1954). In mice only one instance in which heterozygous F_1 grafts survive significantly longer than the corresponding interstrain grafts has been reported (Prehn and Main, 1954). The demonstration of a dosage effect for histocompatibility genes may be possible only under certain conditions, because the amount of antigen produced is not necessarily the same for each histocompatibility gene. In the mouse the so-called strong histocompatibility gene (Counce *et al.*, 1956; Snell, 1957), which elicits a rapid homograft rejection, may control the production of a large amount of antigen, while the so-called weak histocompatibility genes, which elicit less violent and more prolonged reactions, may control the production of smaller amounts. When host and donor differ at a strong histocompatibility locus, sufficient antigen may be produced by a single allele to bring about rapid homograft rejection. If this is a maximum response, differences between the heterozygous and homozygous conditions cannot be detected, and no dosage effect can be demonstrated (FIGURE 8). Similarly, if host and donor differ at a large number of weak histocompatibility loci, the additive effect of these genes may result in an antigenicity sufficiently high to cause a rapid homograft reaction and, conceivably, the antigenicity may reach a level sufficiently high for rapid rejection even when all the histocompatibility genes are present in a heterozygous condition. In such cases it would also be impossible to demonstrate a dosage effect. A similar explanation has been suggested by Prehn and Main (1954). This may account for the failure of Billingham *et al.* (1954) and of Barnes and Krohn (1957) to demonstrate the dosage effect in mice. Indeed, in the latters' experiments, 15 or more histocompatibility genes may have been involved, as indicated by the small number of successful P_1 grafts in the F_2 hosts.

TABLE 20

A COMPARISON OF THE RATE OF BREAKDOWN OF P_1 GRAFTS IN BC AND F_2 HOSTS

Per cent grafts rejected	Strain 30 grafts			Strain 163 grafts		
	Host Bc 816 I	Host Bc 816 III	Host F_2	Host Bc 847 I	Host Bc 847 III	Host F_2
50	14*	17	13	17	15	17
75	20	23	29	22	26	40
87.5	32	34	56	31	33	70

* Days after transplantation, determined by interpolation.

The demonstration of a dosage effect for histocompatibility genes is aslo reminiscent of the work on various human blood group antigens where similar quantitative relationships between gene dosage and antigenicity have been shown to exist (Landsteiner and Levine, 1927; Hill *et al.*, 1950; Lawler and Race, 1950; Race *et al.*, 1943, 1953).

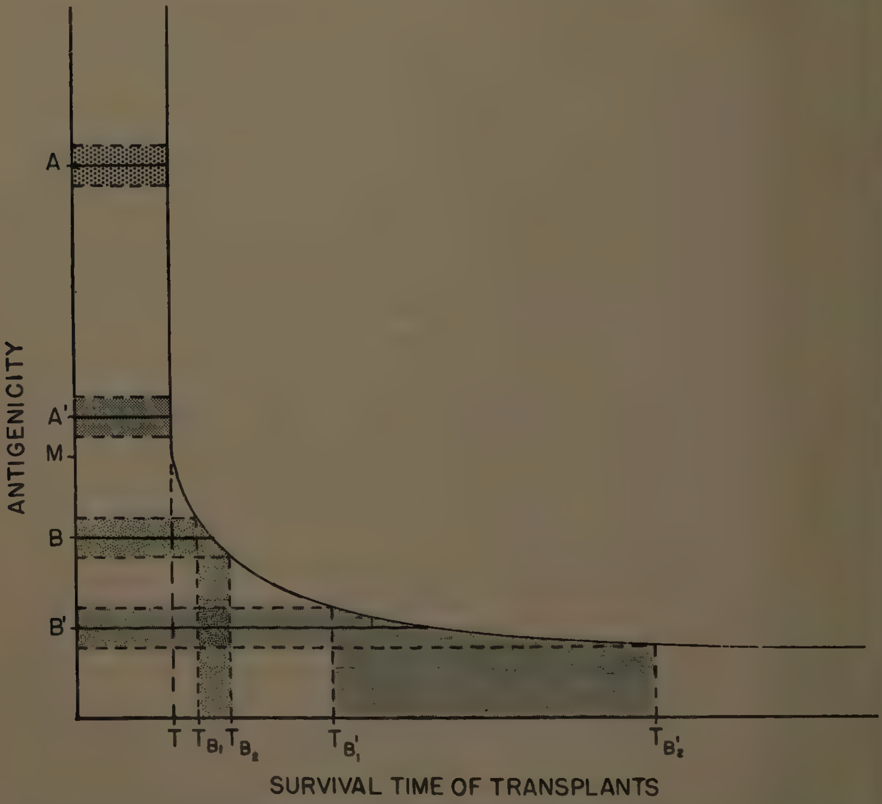

FIGURE 8. Curve showing the relationship between antigenicity and survival times of transplants. A represents the average antigenicity of strongly antigenic homozygous inter-strain grafts. Their antigenicity is well above the level M for maximum response.

A′ represents the average antigenicity of the corresponding heterozygous F_1 grafts. Their antigenicity, although lower than that of A, is still above the level M. Therefore, no difference in the survival time T of homozygous and heterozygous grafts can be detected.

B represents the average antigenicity for weakly antigenic homozygous grafts; B′ the average antigenicity of the corresponding heterozygous F_1 grafts. The antigenicity for both types is well below the level M for maximum response, and the F_1 grafts survive significantly longer from ($T_{B'_1}$ to $T_{B'_2}$) than the homozygous grafts (from T_{B_1} to T_{B_2}).

Slight variations in the antigen level (*shading and broken lines*) of grafts of identical genotype have no or only a small effect upon survival times if the antigenicity is above (A, A′) or near (B) the level M. However, in weakly antigenic grafts (B′) slight variations in antigenicity have a profound effect and cause a wide range of survival times (from $T_{B'_1}$ to $T_{B'_2}$).

In summary, the speed of homograft rejection depends upon the graft's antigenicity which, in turn, is controlled by the number of histocompatibility genes present in the cells of the transplant but absent from the cells of the host.

Dosage and additive effects can be demonstrated in those host-donor systems in which the antigenicity of the grafts is below the level for maximum response. Above a certain level of antigenicity the speed of the homograft reaction is more or less constant.

Two Components of the Homograft Reaction

An analysis of the rejection times of primary and secondary F_1 and P_1 grafts shows that the homograft reaction can be resolved into at least 2 components: (1) the interaction of antibodies and antigens within the cells of the graft, and (2) the rate of production by the host of antibodies against the introduced foreign transplantation antigens. Both, however, are dependent upon the number of foreign histocompatibility alleles in the cells of the graft and, probably, upon environmental factors.

Although little is known about the immediate causes for homograft destruction, it appears likely that the action of the antibodies is upon some important cell constituent and that an antigen-antibody complex is formed that interferes with some metabolic activity of the cells of the graft. If each histocompatibility allele controls the production of a limited quantity of specific antigen, then the number of specific antigen-antibody complexes that can be formed will be a primary function of the number of foreign histocompatibility alleles in the cells of the graft. If host and donor differ by several histocompatibility genes, a much larger amount of antigen will be available in the cells of the graft for the formation of antigen-antibody complexes than in a host-donor combination that differs only at a single locus.

Primary interstrain grafts are rejected within 1 to 2 weeks, while secondary interstrain grafts rarely survive more than 5 days. A period of about a week apparently is necessary for the host to manufacture a sufficiently high titer of antibodies against newly introduced transplantation antigens. Secondary grafts are rejected much faster, because the antibodies in question are already present or the antibodies can be produced more readily at the time of the secondary transplant's implantation into the sensitized host. In the present experiments we found that secondary interstrain grafts were rejected faster than secondary F_1 grafts. Since these 2 types of grafts are exposed at the same time to the same amount of antibody in the sensitized host, differences between the survival times of these secondary P_1 and F_1 grafts could be the result only of differences in the antigen-antibody interactions within the cells of the grafts. It is contended that in the cells of the F_1 grafts fewer antigen-antibody complexes are formed and that, therefore, the metabolic activities of these cells are less drastically interfered with and consequently they survive longer.

When a fin from strain 163 and another from an F_1 donor are simultaneously transplanted into a strain 30 host both grafts are exposed to the same amount of antibody at the same time. The longer survival of the F_1 transplant appears to be the result of the smaller number of antigen-antibody complexes formed. The difference between the survival times of the simultaneously transplanted F_1 and the P_1 grafts in hosts of series Bc 816 I and Bc 847 I must also have resulted from the different numbers of antigen-antibody complexes formed within the cells of these 2 types of grafts.

The rate of antibody production is involved in the longer survival times of single F_1 grafts in hosts of series Bc 816 II and Bc 847 II than of F_1 grafts made simultaneously with P_1 grafts in hosts of series Bc 816 I and Bc 847 I. Since the average number of antigen-antibody complexes that could be formed within the cells of the F_1 grafts was the same, whether the fins were implanted into the Bc I or Bc II hosts, the difference between the survival times of the F_1 grafts in these 2 series must be the result of differences in the rate of antibody production by the hosts. The P_1 grafts of series Bc 816 I and Bc 847 I must have brought about the production of antibody much more rapidly; therefore, the F_1 grafts of the Bc I series were exposed to a high level of antibody sooner than the F_1 grafts of series Bc II.

Finally, the differences between the survival times of the F_1 grafts of series Bc 816 II and Bc 847 II, on the one hand, and the P_1 grafts of series Bc 816 I and III and Bc 847 I and III, on the other, must be attributed both to a delay in antibody production and to a different number of antigen-antibody complexes.

The difference between the survival times of the F_1 grafts of series Bc 816 II and Bc 816 I was much more pronounced than the difference between the survival times of the F_1 grafts of series Bc 847 II and Bc 847 I. The smaller difference between the latter 2 series was probably brought about by the higher temperatures to which these hosts were exposed during the first 3 months after the operation. Evidence is accumulating that the dosage effect is exaggerated at low temperatures, and that it becomes rather small at higher temperatures (Kallman, unpublished experiment). Hildemann (1957) demonstrated that the survival time of homografts in the goldfish was temperature-dependent.

Incomplete Penetrance of Histocompatibility Genes

In the present experiments the percentage of successful F_1 grafts in Bc hosts (22 per cent) was more than twice as great as the percentage of successful P_1 transplants (10 per cent). Furthermore, the percentage of surviving F_1 grafts was reduced to 17 per cent when the hosts simultaneously received a P_1 graft. From these results only one conclusion is possible, namely, that some F_1 grafts survived permanently in spite of the fact that their cells possessed at least one foreign histocompatibility gene.

What enabled these F_1 grafts to survive? If each histocompatibility gene controls the production of only a small amount of antigen, then the presence of a single foreign gene may make the graft so weakly antigenic that it does not stimulate the host to produce a sufficient amount of antibodies, or the number of antigen-antibody complexes formed within the cells of the graft may be insufficient to destroy it. About 7 per cent of the F_1 grafts of series Bc I survived permanently despite the fact that their corresponding P_1 grafts were rejected. It is believed that in the cells of these F_1 grafts not enough antigen was present to form a sufficient number of antigen-antibody complexes to kill the graft. These grafts, therefore, survived in spite of the presence of antibodies.

The total percentage of successful F_1 grafts in series Bc II was 5 per cent higher even than in series Bc I. Since the average number of antigen-antibody complexes that could be formed within the cells of the F_1 grafts of both series was the same, the higher percentage of successful transplants in the Bc II hosts must have resulted from the inability of a number of the F_1 grafts with foreign histocompatibility genes to stimulate their hosts to produce sufficient amounts of antibodies. In Bc I hosts, such grafts would have been rejected, since they would have been exposed to antibodies induced by the concomitantly implanted P_1 graft.

These experiments resemble those of Snell (1958), which showed that tumor transplants in mice could transgress genetic differences at certain weak histocompatibility loci. When the hosts had been previously injected with normal cells from the prospective donor or donor strain, however, a state of immunity was induced and identical tumor transplants were rejected. A similar method was employed by Amos *et al.* (1955). Unlike the experiments of Snell and Amos and their collaborators, immunizing grafts (P_1) in the present study were not implanted prior to the weakly antigenic grafts (F_1), but simultaneously. Moreover, transplants of normal tissue were used. One other case for normal tissues of incomplete penetrance of a histocompatibility gene has clearly been detected through the survival of grafts of normal tissues. In many inbred strains of mice a high percentage of transplants from male donors to female recipients is rejected. In some strains, however, a number of these transplants survive permanently. The histocompatibility allele responsible appears to be located on the Y chromosome (Bernstein *et al.*, 1958; Eichwald *et al.*, 1957, 1958; Sachs and Heller, 1958; Short and Sobey, 1957), but one cannot rule out the possibility, as Fox (1959) has shown recently, that the presence of these sex-specific antigens is the result of the different number of X chromosomes present in host and donor.

Variability of Homograft Survival Times

Histocompatibility genes in these fish apparently produce small amounts of antigen. The expressivity (that is, the amount of antigen produced) of a particular allele may vary slightly. Therefore, some grafts may possess slightly greater amounts of antigens than others from donors of identical genotype. Grafts with smaller amounts of antigen are those that survive permanently or that greatly extend the range of survival times.

Interstrain grafts showed little variation, and all grafts disintegrated within 8 or 9 days. In contrast, some of the F_1 grafts in P_1 hosts were rejected as early as day 8 after transplantation; others survived until day 67. Since all F_1 grafts possessed the same genotype, and all P_1 hosts belonged to a highly inbred line, genetic differences can be ruled out as a cause for these large variations.

This observation may seem to be an apparent contradiction to the well-established principle that physiological traits vary less among F_1 hybrids than among pure lines (Lerner, 1954; Grueneberg, 1954). The lower variability of the survival times of the strongly antigenic P_1 grafts is consistent, however, with the hypothesis that the speed of graft rejection is dependent upon the amount of antigen introduced by each graft and that, once a certain level of antigenicity is reached, a further increase in the antigenic nature of a graft does not accelerate its breakdown. The antigenicity of interstrain grafts was undoubtedly above the threshold for maximum response and, within limits, variations in the amounts of antigen present did not affect appreciably the survival times of these grafts. On the other hand, the antigenicity of the F_1 grafts was below the threshold, and even a slight variation in their antigenic content presumably led to acceleration or delay in their breakdown (FIGURE 8).

In mice a similar relationship between antigenicity and the length and variability of the survival times of homografts can be detected. Grafts between hosts and donors carrying different alleles at the strong histocompatibility locus *H-2* possess a mean survival time of about 10 days, and all grafts are rejected within a relatively short period. On the other hand, when hosts and donors differ at the relatively weak loci *H-1* and *H-3*, the transplants possess a mean survival time of several weeks, and the survival times of individual grafts vary by several weeks or months (Counce *et al.*, 1956). Similarly, Prehn and Main (1954) found that, in mice, skin transplants from homozygous BALB/c donors into DBA/2 recipients were rejected between day 18 and day 42, while the presumably weaker antigenic F_1 (BALB/c × DBA/2) grafts into DBA/2 hosts exhibited a high variability in their survival times, being rejected as early as day 30, others surviving for 200 days or longer.

The mean survival time of skin grafts transplanted from males to females depends upon the strain of mice used. Here, too, one finds that, in those host-donor combinations in which the mean survival time of the grafts is low, all transplants are rejected within a relatively short time, while in those host-donor combinations in which the mean survival time of the grafts is long, the survival times of individual transplants differ by several weeks or months; some grafts even survive permanently (Billingham *et al.*, 1958; Bernstein *et al.*, 1958; Eichwald *et al.*, 1958; Sachs and Heller, 1958; Short and Sobey, 1957). It does not seem justifiable to attribute the variations in the survival times of weakly antigenic grafts to differences in experimental technique.

Length of Homograft Reaction

The action of antibodies may be a subtle one. In most P_1 and F_1 transplants that survived for several months in Bc or F_2 hosts, the homograft reaction was a very protracted affair, sometimes extending over several weeks or even months. The first sign of an incipient homograft reaction in these grafts was a cessation of growth, no destructive changes being visible. This was followed by a gradual disintegration of the cells of the transplants. These results are in agreement with those of Prehn and Main (1954) and of Counce *et al.* (1956), who found that in mice the homograft reaction against a graft that differs only slightly in antigenic make-up from its host extends over several weeks, as compared to a few days when the antigenic difference is great. These results, however, do not support the finding of Barnes and Krohn (1957) that the homograft reaction in weakly antigenic mouse grafts proceeds just as rapidly as in interstrain grafts.

Fin transplants in the platyfish appear to serve as much more sensitive indicators of the beginning of the homograft reaction than do mouse skin grafts. In the latter, the homograft reaction for weakly antigenic transplants lasts for from 20 to 30 days, as judged by the appearance of discoloration, scabs, and ulcerations. In our fin grafts the first sign of a homograft reaction is not the destruction of tissue, but a cessation of growth or regeneration, and this frequently manifests itself 2 to 3 months before final destruction of the graft.

SUMMARY AND CONCLUSIONS

The teleost *Xiphophorus maculatus* is uniquely suited to extend our knowledge of the genetics of transplantation to lower vertebrates, since (1) it is the only poikilotherm in which inbred strains are available, (2) it can be bred and raised in small aquaria and is quite hardy, and (3) transplants can be made easily, rapidly, and with consistent results. Two genetically related, highly inbred strains (30 and 163) and their F_1, F_2, F_3, and Bc hybrids were used.

All intrastrain grafts and all but 1 of many transplants from P_1, F_1, F_2, and F_3 donors survived in F_1 hosts. All interstrain grafts and all grafts from F_1 donors to P_1 hosts were rejected. Second-set interstrain grafts were rejected at an accelerated rate. Second-set F_1 grafts were rejected more rapidly than first-set F_1 grafts, but they survived longer than second-set interstrain grafts.

The number of histocompatibility genes involved in determining the fates of transplants between the 2 inbred strains was estimated from the percentages of successful P_1 grafts in hosts of the F_2 and Bc generations. These results indicate that approximately 3 histocompatibility genes are involved.

The rate of rejection of P_1 grafts in F_2 and Bc hosts was considerably slower than that of interstrain grafts. Since many F_2 and Bc hosts differ from their P_1 grafts in only 1 or 2 histocompatibility genes, the rapid rejection of interstrain grafts must be due to an additive effect of histocompatibility genes.

In all experiments F_1 grafts survived significantly longer than did P_1 grafts. Since F_1 and P_1 donors possess the same histocompatibility genes, the genetic difference between them being solely one of dosage, the faster rejection of P_1 grafts must result from a dosage effect of the histocompatibility genes.

The percentage of successful F_1 grafts in hosts of Bc generations was twice as high as the percentage of successful P_1 grafts. The percentage of accepted F_1 grafts could be lowered significantly by simultaneously transplanting a P_1 graft with the F_1 graft. In some of these hosts only the P_1 graft was rejected, while the F_1 transplant survived permanently. Since both F_1 and P_1 donors possess the same histocompatibility genes, some F_1 grafts must survive even though they possess at least one foreign histocompatibility gene.

Two components of the homograft reaction can be distinguished, one concerned with the interaction of antibodies and antigens inside the cell of the graft, the other with the rate of antibody production by the host. The magnitude of both components is dependent upon the number of histocompatibility alleles present in the cells of the graft but absent from the cells of the host.

The homograft reaction in interstrain grafts lasted from 2 to 5 days, while the homograft reaction of P_1 grafts in many F_2 and Bc hosts lasted for several weeks or months. This reaction was first indicated by a general retardation of the transplants' growth or a lack of regeneration.

These experiments suggest that each histocompatibility gene controls the production of a certain quantity of specific antigen and that, within limits, the survival time of a graft is related to the amount of foreign antigen introduced. Once a certain level of antigenicity is reached, however, homograft rejection proceeds at maximum speed, and a further increase in the antigenicity of a graft does not lead to faster rejection. The concept that the presence of histocompatibility genes in the graft and their absence in the host leads to a homograft reaction which eventually destroys the graft is supported only partially by these experiments. According to this concept, the percentages of successful F_1 and P_1 transplants in the hosts of the Bc generation should be the same. Because of the significantly higher percentage of surviving F_1 grafts in Bc hosts and the prolonged survival of many other grafts that differed from their hosts by only 1 or 2 histocompatibility genes, it is concluded that these genes control the production of limited amounts of antigen. Grafts that differ from their hosts by only a single histocompatibility gene are sometimes so weakly antigenic that they fail to elicit a homograft reaction, and these grafts survive indefinitely. Unless special methods are employed, the transplantation test may fail to reveal a difference at a weak histocompatibility locus.

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References

Amos, D. B., P. A. Gorer & Z. B. Mikulska. 1955. The antigenic structure and genetic behavior of a transplanted leukosis. Brit. J. Cancer. **9:** 209–215.

Barnes, A. D. & P. L. Krohn. 1957. The estimation of the number of histocompatibility genes controlling the successful transplantation of normal skin in mice. Proc. Roy. Soc. London. **B146:** 505–526.

Bernstein, S. E., A. A. Silvers & W. K. Silvers. 1958. An attempt to demonstrate a Y-linked histocompatibility gene in the house mouse. J. Natl. Cancer Inst. **20:** 577–580.

Billingham, R. E., L. Brent, P. B. Medawar & E. M. Sparrow. 1954. Qualitative studies on tissue transplantation immunity. I. The survival time of skin homografts exchanged between members of different inbred strains of mice. Proc. Roy. Soc. London, **B143:** 43–58.

Billingham, R. E. & W. K. Silvers. 1958. Induction of tolerance of skin isografts from male donors in female mice. Science. **128:** 780–781.

Breyere, E. J., Jr. 1957. Several immunogenetic responses of the rat as determined by skin transplantation. Dissertation Abstr. **17:** 2386.

Counce, S., P. Smith, R. Barth & G. D. Snell. 1956. Strong and weak histocompatibility gene differences in mice and their role in the rejection of homografts of tumors and skin. Ann. Surg. **144:** 198–204.

Eichwald, E. J., C. R. Silmser & N. Wheeler. 1957. The genetics of skin grafting. Ann. N. Y. Acad. Sci. **64**(5): 737–740.

Eichwald, E. J., C. R. Silmser & I. Weissman. 1958. Sex-linked rejection of normal and neoplastic tissue. I. Distribution and specificity. J. Natl. Cancer Inst. **20:** 563–575.

Fox, A. S. 1959. Genetic determination of sex-specific antigens. J. Natl. Cancer Inst. **23:** 1297–1311.

Gordon, M. 1950. Fishes as laboratory animals. *In* Care and Breeding of Laboratory Animals. **14:** 345–449. E. J. Farris, Ed. Wiley. New York, N. Y.

Goss, R. J. & M. W. Stagg. 1957. The regeneration of fins and fin rays in *Fundulus heteroclitus*. J. Exptl. Zool. **136:** 487–508.

Grueneberg, H. 1954. Variation within inbred strains of mice. Nature. **173:** 674–676.

Hildemann, W. H. 1957. Scale homotransplantation in the goldfish (*Carassius auratus*). Ann. N. Y. Acad. Sci. **64**(5): 775–791.

Hill, J. M., S. Haberman & F. Jones. 1950. The *Rh* antigen mosaic of erythrocytes. The effect of gene dosage shown by quantitative hemolytic studies. Proc. Intern. Soc. Hematol. **3:** 179–187.

Kallman, K. D. & M. Gordon. 1957. Transplantation of fins in xiphophorin fishes. Ann. N. Y. Acad. Sci. **71**(3): 305–320.

KALLMAN, K. D. & M. GORDON. 1958. Genetics of fin transplantation in xiphophorin fishes. Ann. N. Y. Acad. Sci. **73**(3): 599–610.

KALLMAN, K. D. Gynogenesis in the teleost, *Poecilia formosa* (Girard), with a discussion of the detection of parthenogenesis in vertebrates by tissue transplantation. Genetics. In press.

LANDSTEINER, K. & P. LEVINE. 1927. Further observations on individual differences of human blood. Proc. Soc. Exptl. Biol. Med. **24:** 941–942.

LAWLER, S. D. & R. R. RACE. 1950. Quantitative aspects of *Rh* antigens. Proc. Intern. Soc. Hematol. **3:** 168–170.

LERNER, I. M. 1954. Genetic Homeostasis. : 1–134. Oliver & Boyd. London, England.

PREHN, R. T. & J. M. MAIN. 1954. A comparison between heterozygous and homozygous skin homografts. J. Natl. Cancer Inst. **15:** 191–194.

PREHN, R. T. & J. M. MAIN. 1958. Number of mouse histocompatibility genes involved in skin grafting from strain BA1b/cAn to strain DBA/2. J. Natl. Cancer Inst. **20:** 207–209.

RACE, R. R., R. SANGER & D. LEHANE. 1953. Quantitative aspects of the blood-group antigen Fy^a. Ann. Eugenics. **17:** 255–266.

RACE, R. R., G. L. TAYLOR, K. E. BOORMAN & B. E. DODD. 1943. Recognition of *Rh* genotypes in man. Nature. **152:** 563–564.

SACHS, L. & E. HELLER. 1958. The sex-linked histocompatibility antigens. J. Natl. Cancer Inst. **20:** 555–561.

SHORT, B. F. & W. R. SOBEY. 1957. The effect of sex on skin grafts within inbred lines of mice. Transplantation Bull. **4:** 110–112.

SNELL, G. D. 1957. The genetics of transplantation. Ann. N. Y. Acad. Sci. **69**(3): 555–560.

SNELL, G. D. 1958. Histocompatibility genes of the mouse. I. Demonstration of weak histocompatibility differences by immunization and controlled tumor dosage. J. Natl. Cancer Inst. **20:** 787–824.

DISCUSSION OF THE PAPER

E. J. EICHWALD (*Montana Deaconess and Columbus Hospitals, Great Valley, Mont.*): I was impressed by Kallman's courage in speaking of antibodies in fish, and I wonder if he speaks of such bodies in an actual or in a hypothetical sense.

KALLMAN: We have never demonstrated the antibodies, but we believe that they must be there.

D. B. AMOS: I think you would be safer if you spoke of an immune state in fish rather than an antibody, which is still hypothetical. One thing that was not clearly presented and that concerned me throughout the paper was that, although you gave plenty of data for most of the transplantations you performed, you did not give much control data. The variations in behavior of the transplants from the F-1 line made me wonder whether the F-1s were truly from strictly homozygous parents, as this is the sort of result that is obtained if there is some heterozygosity in the two parental strains. Your remarks about the variation of antigenicity or the strength of different antigens are very interesting. This variation does not depend necessarily upon the amount of antigen formed. It probably depends upon the reactive grouping of these antigens, since I am sure that you recognize that even simple substitutions make a tremendous difference in the effectiveness of the antigen.

I agree with you when you say that in the mouse there are tremendous differences between different histocompatibility systems, and this is almost certainly (or seems to be from your data) the same situation in the fish.

MERRILL W. CHASE: The basic assumption, probably quite valid, made here is that the degree of antibody response reflects proportionately the total antigenic charge or burden borne by the recipient, but from the general experiences of those who have injected rabbits with pure antigens, alternative principles develop. One of these is that under conditions of exposure to multiple antigens one may obtain distribution in responses, and that one antigen is not equivalent to another antigen.

We are confronted with graded kinds of responses to antigens, and we see this principle in the Rh family of antigens, if we turn to the human species. Some antigens are much "stronger" than others.

Therefore, I should think that, if our first concept is of antigen-antibody relationships, we could think not only of the burden in terms of numbers of antigens but also in relative weightings as effective antigens that we might have to assign to them; this might involve minor changes in the general pattern of interpretation.

I repeat the second point that Amos has already made: the opinion one might entertain as to why a graft is rejected could have other bases than assigning the sole role to antigen-antibody complexes and a search for circulating antibody. One can only make an observation, as the author has, and then seek its explanation.

EICHWALD: Most of us, of course, are not geneticists. I ask this question: Is it not true that, according to the rules of classic genetics, it should not make any difference whether an antigen in a situation such as this is homozygous or heterozygous, as long as it is there? Is that not the difference between the genotype and the phenotype?

R. T. PREHN (*National Cancer Institute, Public Health Service, Bethesda, Md.*): We published some data on mice a few years ago that showed there was just such a dilution effect, at least in some genetic combinations. Therefore, it is not always true that the heterozygous gene will act in the same manner as a homozygous gene. I think this is quite apparent.

EICHWALD: Yes, but what is the explanation for that?

PREHN: That is another story.

KALLMAN: We have done some experiments in other species of fish, where we have also been able to demonstrate a dosage effect whenever the host and donor are closely related. However, when we use the host-donor system where the host and donor come from completely different strains, or if we use two inbred strains belonging to different species, then we cannot demonstrate a dosage effect.

This has led me to the conclusion that a dosage effect can be demonstrated only when the level of antigenicity is not too high.

SEXUAL DIMORPHISM IN THE HISTOCOMPATIBILITY REACTIONS OF AMPHIBIA TO SKIN HOMOGRAFTS AND A TENTATIVE EXPLANATION OF THEIR MECHANISM*

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Introduction

Recent experimental work (Eichwald *et al.*, 1957; Billingham, 1958) has shown that in mammals there are histocompatibility factors evidently associated with the Y chromosome that are responsible for the tolerance or rejection of isografts by the host.

It was shown that when isografts were made in certain mice considered genetically identical but differing only in sex, the grafts were sometimes rejected. This occurred, however, only when the donor was male and the host female. When the female was the donor and the male the host, no rejection was noted. Since the animals differed from each other only in sex, it has been supposed that the isografts were rejected or retained because of differences in an antibody response of the host to factors of the graft that existed only on the Y chromosome, that is, only in the male. These factors were called histocompatibility or histoincompatibility factors.

Hauschka recently has noted (personal communication, 1958) that a crossover between the X and Y chromosomes may take place involving these factors so that in some instances females will have the factors.

Work in our laboratory has indicated that a similar mechanism exists in the newt *Triturus viridescens*. In this animal, however, the factors are probably responsible for the rejection of skin homografts and not only for isograft rejection, as in mammals. Other evidence tends to indicate that the female also plays a role in the phenomena of homotransplantation. In the following a brief summary is given of experiments and their main results, the details of which will be published elsewhere (Pizzarello, 1960). This is followed by a discussion and a tentative explanation of the findings.

Material and Methods

Newts of the species *T. viridescens* were used in all cases. The populations used were genetically completely random. Some were collected in New York State and some in North Carolina. It is interesting to note that no differences were found in the results when the two groups were compared with each other or when they were used in intrageographical transplantation.

The technique of grafting was a very simple one that required neither suturing nor bandaging. The graft bed was prepared by making a slit in the skin of the host animal between the lateral processes of the vertebrae. The graft then was pressed firmly into the graft bed with the epidermis up. The fit is

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quite tight so that the graft remains well in position. By this method, from one to ten grafts may be placed in a single host (FIGURE 1). The grafts were marked either by natural color differences (light belly skin grafted on the olive-green dorsal side of the host) or by applying a vital stain (1:200,000 aqueous solution of neutral red) to the donor for five days before transplantation.

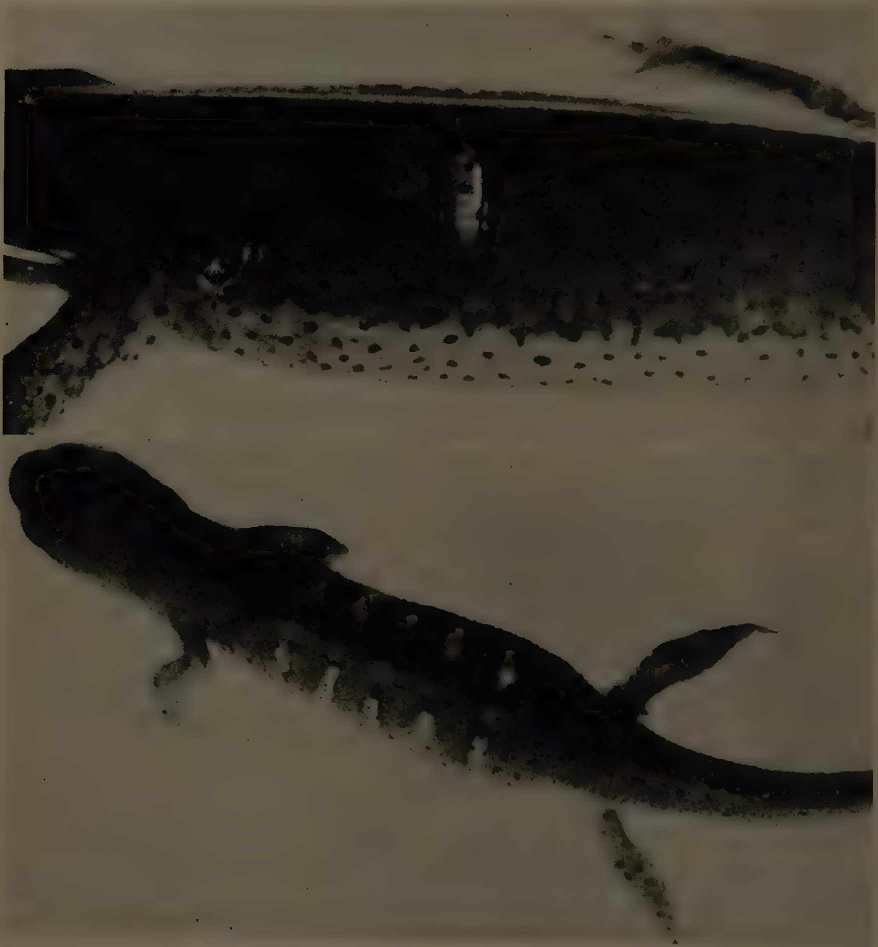

FIGURE 1. (*Top*), single graft of belly skin to dorsal side of host. *Bottom*, multiple grafts (10 implants of belly skin) to dorsal side of host.

Two types of implants were made. One consisted of the epidermis and the mucous glands ("single-thickness implant"), the other of the dermis and subepidermal connective tissue, muscle, and fascia, as well as the epidermis "full-thickness implant"). No difference in results was noted with the two types.

Two experimental series were studied with these techniques. In one only a single small implant of either the full- or single-thickness type was grafted to an individual host ("single implants"). In the other series, from two to ten implants of either the full- or single-thickness type were grafted to an individual host ("multiple implants").

All combinations were made, that is, male-to-male, male-to-female, female-to-male, and female-to-female, in both the single and multiple series.

Observations and Results

As already stated, no difference in results was seen between single-thickness and full-thickness grafts. These categories, then, will not be treated separately.

Single-implant series. In these the homografts that persist the longest are the female-to-female and female-to-male. The male-to-male and male-to-female are rejected quickly. There was one exceptional case (a male graft that persisted in a male host for 56 days) that will be discussed later. The female-to-female and female-to-male homografts were tolerated for more than 100 days by the host. The male-to-male and male-to-female were tolerated only 15 days. Preliminary data on this series already have been communicated in discussion at the Third Homotransplantation Conference of The New York Academy of Sciences (1958) and in a brief note (Pizzarello and Wolsky, 1958).

Multiple-implant series. As the number of implants in a single host is increased, the tolerance of the hosts is exactly opposite from that in the single-implant series. The grafts that persist the longest in this series are the multiple male-to-male implants. These, in fact, are maintained 5 times longer than a single male-to-male implant would be. On the other hand, multiple female-to-female grafts are rejected quickly. In such cases, however, there appears to be a certain quantitative aspect. A single female-to-female implant persists more than 100 days. Two female implants in a female host persist 56 days, or about one half of that time. Three or more persist only 10 or 15 days. There appears, then, to be a threshold effect at 3 such implants.

The sex-crossed multiple implants, that is, multiple male-to-female and multiple female-to-male, are sloughed in about 10 days, regardless of the number of implants.

In the multiple-implant series a female bearing 10 female homografts tolerated the implants for 33 days. This exceptional case, together with the exceptional single-implant case mentioned above, are considered under *Discussion and Conclusions.*

Histological observations. Graft rejection in the newt does not differ histologically from that in mammals (Toolan, 1958). In all grafts lymphocytes enter the implant. They proceed through the graft and apparently dissolve it.

The difference between grafts quickly rejected and those tolerated for long periods of time is chiefly one of time. In a graft that is rejected quickly the lymphocytes enter within the first two or three days. By the fourth day the graft is filled completely with these cells (FIGURE 2). By the sixth day nothing remains of the graft except collagen fibers: even the lymphocytes are gone (FIGURE 3).

In grafts that persist for long periods of time the histological picture is not actually different. These grafts are rejected by the host in the same way as are those that are rejected quickly. The difference is one of time. The invasion of lymphocytes may not occur in the graft until six months after implantation. Even then the population is small, and graft lysis is a slow process

(FIGURE 4). Consequently, the host tolerates these implants for a long time, but eventually they also are destroyed.

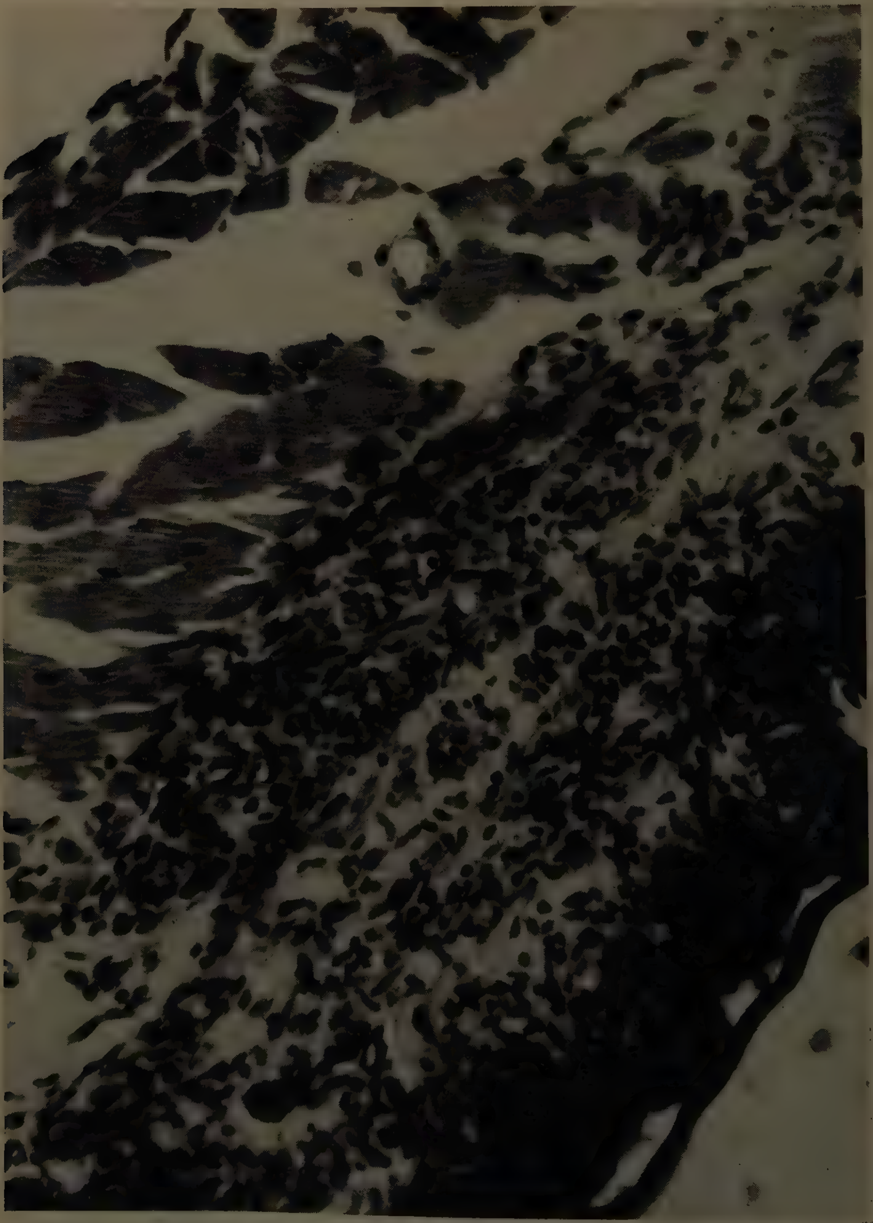

FIGURE 2. Single male-to-male implant 4 days after grafting. Note the prompt and extensive lymphocyte infiltration reaction. Hematoxylin-eosin. ×100.

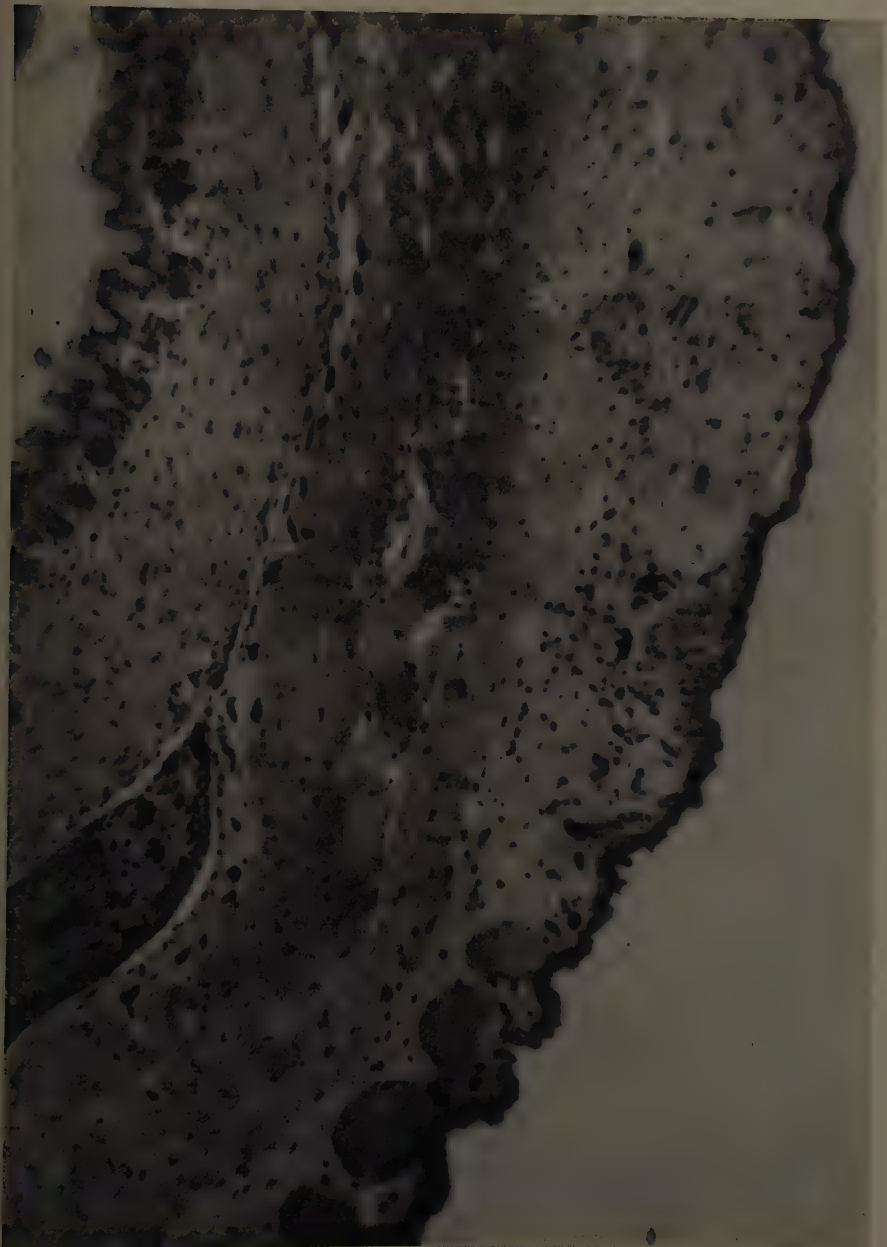

FIGURE 3. Single male-to-male implant 6 days after grafting. The graft is at the *top*. The *bottom* shows normal skin of the host. At this time the lymphocyte reaction has largely disappeared, and the graft is virtually without structure. Hematoxylin-eosin. ×50.

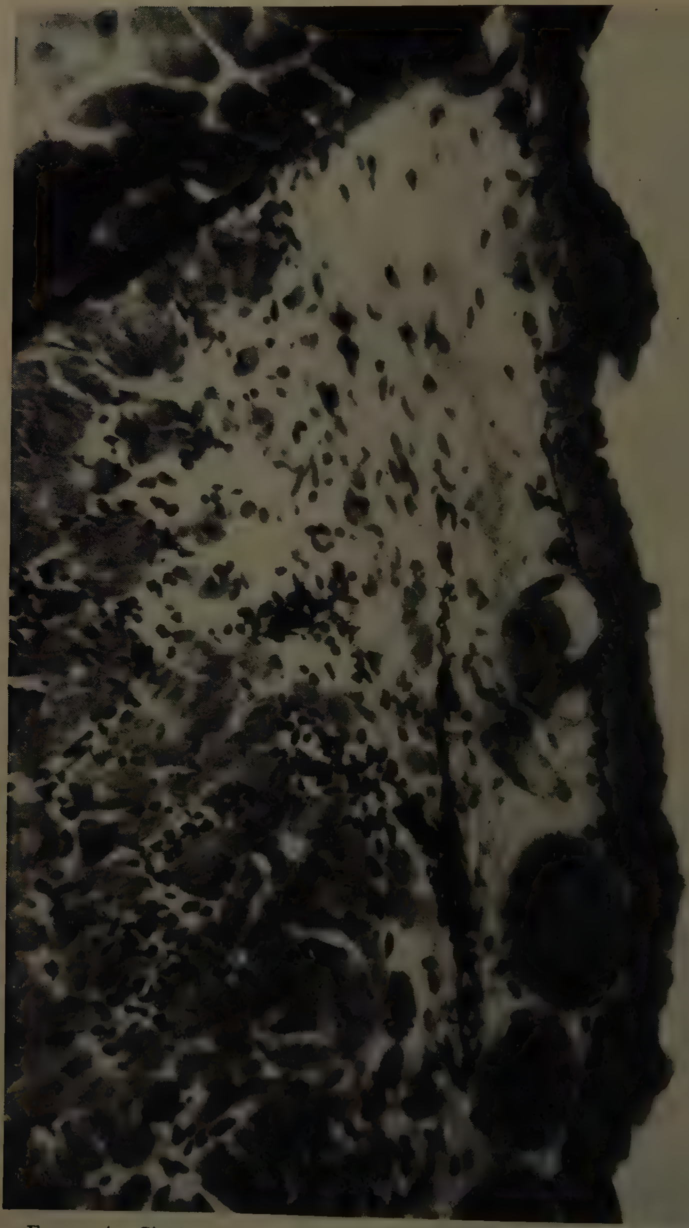

FIGURE 4. Single female-to-female implant 6 months after grafting. Although certain destructions are visible at the top of the figure, the structure of the graft is remarkably intact, including the skin glands and pigment cells. Hematoxylin-eosin. ×100.

Discussion and Conclusions

We believe that these somewhat paradoxical results may be explained on the basis of a few assumptions that accord with established facts of gene physiology and the nature of histocompatibility. We assume that the reactions between host and graft, although essentially immunological in character (antigen-antibody interactions), are not serologic in the strict sense, but take place at a cellular level. Their cytogenetic basis is in histocompatibility genes, which are located in the chromosomes of the nuclei and consist essentially of desoxyribonucleic acid (DNA). The specific DNA of the histocompatibility genes is responsible, through the nucleocytoplasmic ribonucleic acid (RNA) system of corresponding specificity, for the synthesis of specific proteins in the cytoplasm. This concept is strongly supported, in the case of amphibians by studies on hybrid frogs' eggs (*Rana esculenta* ♀ × *R. temporaria* ♂; Brachet, 1952) and on hybrid merogonic newts' eggs (*Triton palmatus* ♀ × *Tr. cristatus* ♂; Zeller, 1956). The specific proteins of the cytoplasm, produced under the control of histocompatibility genes, will act as antigens when the cells are transplanted to a foreign organism and come in contact with cells of the host. The primary visible reaction of the host to the antigen seems to be an activation and quick multiplication of lymphocytes under the stimulus of a chemical reaction between the antigen and its complementary proteins (antibodies) in the cytoplasm of these cells. This reaction may be similar to the fertilization reaction between sperm and egg, which is considered by many to be essentially an immunological reaction (Tyler, 1957). Such a similarity was pointed out recently by Schultz (1959). Since both the antigen and antibody formations take place in the cytoplasm under the control of nuclear genes, the difference in histocompatibility between the male and female tissues must be in the genetic constitution of the two sexes. The simplest assumption is that the difference is quantitative and is due to the presence of histocompatibility genes in the Y chromosome, as postulated recently for mammals by several authors (Eichwald *et al.*, 1957; Billingham, 1958). These genes have hypomorph alleles on the X chromosome or they manifest themselves more strongly on the Y chromosome because of differences in the genic environment. In other words, they may exhibit a "position effect" similar to that demonstrated in *Drosophila* with the cubitus interruptus gene translocated from a euchromatic into a heterochromatic region (Dubinin *et al.*, 1935), or with genes of a quantitative character (ocellar bristle number) having alleles both on the X and Y chromosomes (Mulrennan and Wolsky, 1957; Mulrennan, 1957).

In *Triturus* the sex-determining mechanism is XX-XY (Wickbom, 1945; White, 1946; Clifford, 1949). If it is assumed that in these animals the male (XY) cells have a higher antigen content than do the female (XX), and that the female cells have a higher sensitivity to the antigen, perhaps a higher antibody-synthesizing capacity, than do the male (possibly because of genes with additive effects located on the X chromosomes and with no alleles on the Y chromosomes), a formal explanation may be given of the "paradoxical" results of the single- and multiple-homotransplantation experiments in males and females (FIGURE 5).

The cases in which single grafts failed to cause a prompt rejection (that is, the female-to-female and female-to-male grafts) can be explained by the lack of sufficient antigen in the grafted cells. This in turn would be due to the fact that female cells lack the Y chromosome and its histoincompatibility genes. The small quantity of antigen introduced would not be sufficient to elicit an antibody response either in a female host, which responds vigorously, or in a male host, whose response is weaker.

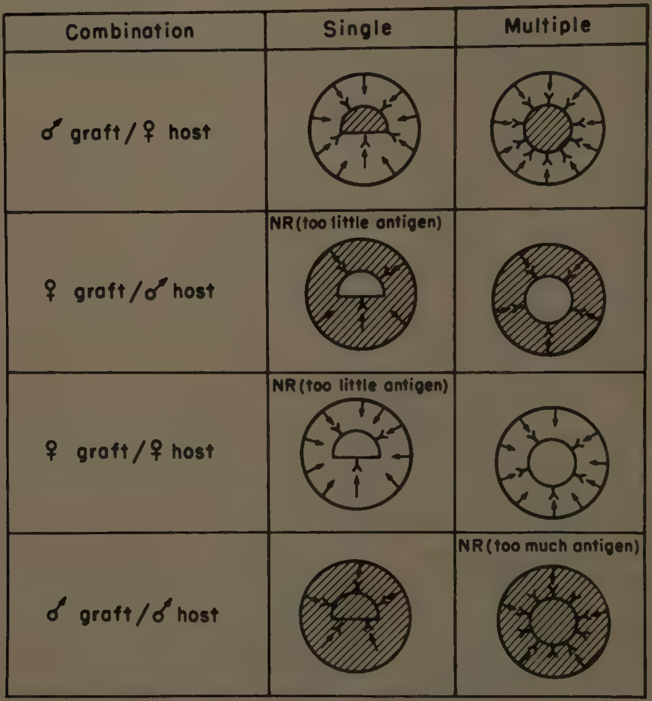

FIGURE 5. Formal explanation of the results of skin grafts. The Y signs on the inner circle of the figures represent the antigen content of the graft, the arrows in the outer circle represent the antibody content of the host. The density of the Ys and arrows indicates quantitative differences: 5 per full circle means small content, 10 means high content. If there are less than 5 Ys in a graft, there is no rejection reaction because the amount of the antigen is insufficient; where there are more Ys than arrows, "immunological paralysis" is occurring and there will be no rejection reaction because of this. The combinations in which no rejection takes place are marked with NR, and the cause is given. Female tissue (XX), white; male tissue (XY), shaded.

The cases in which multiple skin grafts are better tolerated than are single ones, namely, the male-to-male, may be considered cases of "immunological paralysis" due to massive overdoses of antigen added to a relatively weak antibody system, similar to that described by Tyler (1957) and Tyler and Brookbank (1955; 1956*a*,*b*) concerning the immunological blocking of cleavage in sea urchin eggs.

The quick response elicited by single male grafts either in another male or in a female is interpreted as a result of a sufficient amount of antigen in the male graft due to the presence of Y chromosomes in its cells. The antibody system of the male is sufficient to respond to this amount of antigen, although not to the overdoses administered in multiple grafts. The female, of course, will reject quickly the single male graft because it has a well-developed antibody-synthesizing system associated with the XX constitution of its cells.

The quick response elicited by multiple female grafts in a female host is considered to be due to the additive effect of multiplying a number of small grafts, each having a small amount of antigen and each of which alone would take months to cause its own destruction.

The two exceptional cases in which grafts were tolerated against expectation (one multiple female graft and one single male-to-male graft) may be rare instances in which crossing over has occurred between sections of the X and Y chromosomes. Such crossing-over between X and Y chromosomes was demonstrated experimentally by Winge (1923) in the cyprinodont fish *Lebistes*; the likelihood of its happening in *Triturus* is increased because of the morphologic similarity of the two kinds of sex chromosomes in this genus.

There are ways of testing the assumptions made in this paper, especially by the use of spleen cells. To determine, for example, whether the antibody response of the male host to multiple grafts really breaks down because of an immunological block, as we suggest, one could implant (inject) spleen cells in the male host with a single graft and see if this prevents the rejection reaction as does the multiplication of grafts. Similarly, the rejection reaction of the female host to a single female skin graft could be elicited by a sensitization of the host with spleen cells—provided that our explanation that such a rejection does not occur because there is too little antigen in the female skin graft is correct. Enucleated spleen cells and naked spleen cell nuclei could be used in parallel experiments to determine whether both the nucleus and the cytoplasm are needed or whether either alone can produce the phenomena. The use of spleen cells would also help in determining whether the phenomena we have observed are restricted to skin tissues or whether all cells of the organism behave in the same way.

These and similar approaches would help, we believe, to confirm or to disprove the validity of the tentative explanation proposed in this paper. Studies along these lines are in progress.

References

BILLINGHAM, R. E. 1958. Induction of tolerance of skin isografts from male donors in female mice. Science. **128:** 780–781.

BRACHET, J. 1952. The role of the nucleus and the cytoplasm in synthesis and morphogenesis. Symposium Soc. Exptl. Biol. **6:** 173–200.

CLIFFORD, SR., A. 1949. A comparative study of spermatogenesis in five species of salamanders. Dissertation. Fordham. Univ. New York, N. Y.

DUBININ, N. O., N. N. SOKOLOV & G. G. TINIAKOV. 1935. Cytogenetic study of the position effect. Biol. Zhurn. **4:** 707.

EICHWALD, E. J., C. R. SILMSER & N. WHEELER. 1957. The genetics of skin grafting. Ann. N. Y. Acad. Sci. **64**(5): 737–740.

MULRENNAN, C. A. SR., 1957. The variability of ocellar bristles and their inheritance in *Drosophila melanogaster*, Meigen. M.S. Dissertation. Fordham Univ. New York. N. Y.

MULRENNAN, C. A. SR. & A. WOLSKY. 1957. Hereditary differences in the chaetotaxis of two inbred strains of *Drosophila melanogaster* from geographically isolated areas. Anat. Record. **128:** 593.

PIZZARELLO, D. An experimental study of skin homotransplantation in the newt, *Triturus viridescens* (Raf.). In press.

PIZZARELLO, D. & A. WOLSKY. 1958. Sex relationship in tissue homotransplantation in the newt, *Triturus viridescens*. Anat. Record. **131:** 589.

SCHULTZ, J. 1959. Antigens and antibodies as cell phenotypes. Science. **129:** 937.

TOOLAN, H. W. 1958. Studies of adult and embryonic skin homografts on conditioned or normal rabbits, with emphasis on the possible role of ground substance. Ann. N. Y. Acad. Sci. **73**(3): 546–560.

TYLER, A. 1957. Immunological studies of early development. *In* The Beginnings of Embryonic Development. A Tyler, R. D. von Borstel & C. B. Metz, Eds. Publ. **48.** Am. Assoc. Advancement of Sci. Washington, D. C.

TYLER, A. & J. W. BROOKBANK. 1955. Inhibition of cell division in sea urchin eggs by specific antiserums. Science. **122:** 881.

TYLER, A. & J. W. BROOKBANK. 1956*a*. Antisera that block cell divisions in developing eggs of sea urchins. Proc. Natl. Acad. Sci. U. S. **42:** 304.

TYLER, A. & J. W. BROOKBANK. 1956*b*. Inhibition of division and development of sea urchin antisera against fertilization. *Ibid.* **42:** 308.

WHITE, M. J. D. 1946. The spermatogenesis of hybrid between *Triturus cristatus* and *T. marmoratus* (Urodela). J. Exptl. Zool. **102:** 179–208.

WICKBOM, T. 1945. Cytological studies on dipnoi, urodela, anura, and emys. Hereditas. **31:** 16–346.

WINGE, O. 1923. Crossing-over between the X and Y chromosome in Lebistes. J. Genet. **12:** 2.

ZELLER, A. 1956. Über den Ribonukleinsäure-Stoffwechsel des Bastardmerogons *Triton palmatus* ♀ × *T. cristatus* ♂. Roux Arch. Entw. Mech. **148:** 311.

Discussion of the Paper

W. H. HILDEMANN (*University of California School of Medicine, Los Angeles, Calif.*): The findings of Pizzarello and Wolsky in the salamander are certainly provocative and interesting. These investigators tell me that they have also recently transplanted some second-set grafts in these animals that show the expected accelerated rejection, thereby verifying the immunological basis of the reaction.

My associates and I have recently done some extensive work with bullfrog larvae and adults at various ages, and the sex-specific antigens that play a role in the salamander do not seem to do so in the bullfrog. However, this perhaps is not surprising. It is clear that we know, at present, all too little about the diversity of transplantation responses in the various cold-blooded vertebrates.

I wonder whether the temperature at which these experiments were done was carefully controlled. I presume so because, of course, the survival time can vary very greatly unless the water temperature is thermostatically controlled.

The chief reservation I think one might have in the interpretation of these results is that, while the male-specific antigens seem to be clearly involved in the rejection here and while it is obvious that these male-specific antigens in salamanders are quite strong as judged by the rapid rejections, they contrast with those found in certain inbred mice, which by and large lead to a chronic, very slow rejection.

However, even in mice, insofar as I know, such male-specific antigens really have not been proved to be determined directly by "Y"-linked genes. I think one should keep in mind that we are dealing with the possibility of perhaps autosomal genes that are sex-limited to males or, alternatively, that some gene on the "Y" chromosome starts in motion a developmental process that is impinged upon subsequently by other genes of the organism.

Since the genetic constitution of these animals, as you have pointed out, is not well defined, one would assume they are really quite randomly bred, diverse individuals. I think one should be very cautious in interpreting these results as related to events involving the sex chromosomes.

I wonder, indeed, if the present data might just as well be interpreted in terms of a weak isoantigen present in both males and females, calling to mind again the fact that a single male-to-male graft is rejected rather promptly but that there is, in addition, a stronger antigen or antigens characteristic of males which in large dosage, for example, the use of multiple grafts, might lead to enhanced homograft survival.

PREHN: From a cursory inspection, I can see no more logical explanation of the data cited than that already offered. Certainly, we have many precedents in a wide variety of species for an effect of dosage, for example, the fact that a large dosage of antigen is able to overcome an immunity that would otherwise be present. We have certainly seen this over many years in various kinds of tumor grafts where a small graft will not take and a large one will.

I was also impressed by the facility with which you can show an apparent histocompatibility gene on the "Y" locus. In a situation in which the animals used are not inbred, it is quite apparent that under ordinary circumstances in which there is a large degree of genetic diversity among these animals, one could not hope to demonstrate this sex-linkage effect because it would be largely masked.

Apparently it was believed for many years that histocompatibility relationships were not as important in the cold-blooded animals as in mice. Is this true? Or, is it that these animals are actually fairly homogeneous genetically, because they have all been raised perhaps in a similar pond or for some analogous reason?

PIZZARELLO: May I answer this question by stating that our animals could not have been genetically related, as they were not even from the same state.

EICHWALD: The next paper will be presented by W. H. Hildemann. I noticed with distress that in his abstract there is a term that should arouse concern: namely the word homovital. Many contributors to these pages, I am sure, were present when this term was born, and I do not suppose the obstetrician should be blamed if the child is a malformation; but one often regrets the survival of malformations, and I wonder if Hildemann will now or later say something in justification of this term.

CHRONIC SKIN HOMOGRAFT REJECTION IN THE SYRIAN HAMSTER*

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Introduction

Recent studies with various random-bred but closed colonies of Syrian hamsters showed this species to be exceptional in that intracolony as well as certain intercolony skin homografts survived for many months with no evidence of incompatibility.[1] However, in four of six *intercolony* combinations tested, most animals rejected their homografts in the usual manner after acute reactions. This finding indicated that hamsters are potentially capable of rejecting homografts as vigorously as other mammals. In the above experiments, nevertheless, a number of intracolony as well as intercolony homograft exchanges resulted in chronic reactions with delayed onset and a prolonged period of rejection. Such chronic skin homograft breakdown commencing after many months of apparent full viability, was noted also by Adams *et al.*[2] in presumably random-bred Syrian hamsters from two separate stocks.

Among several inbred lines of mice, Counce *et al.*[3] found that skin grafted between animals differing at "weak" histocompatibility loci (H-1 and H-3) showed prolonged survival, while a disparity at the "strong" H-2 locus invariably led to rapid destruction of the grafts. The protracted rejection of isologous male to female grafts observed in many inbred lines of mice is clearly elicited by weak male-specific antigen(s).[4,5] It is now also established that chronic skin homograft rejection may occur in rats,[6,7] mice,[8] chickens,[9] and frogs[10] that have been made incompletely tolerant toward homograft donors. Nevertheless, chronic homograft rejection as such has apparently not been investigated in detail in any species heretofore.

The present study involved adult Syrian hamsters from a closed but outbred colony in which initial prolonged homograft acceptance was regularly observed to be followed by a variable process of slow rejection. The various experiments were planned to determine and correlate the immunological and histopathological characteristics of the chronic homograft reaction in this species.

Materials and methods. The hamsters employed were obtained from a large colony maintained by the Bezanson Hamstery, Temple City, Calif. This colony was started with about 30 animals and has been closed for 10 years. Individual breeders have been taken only from litters of 10 or more animals, the offspring of separate sets of parents. Continued outbreeding with selection for fertility has been assured by the use of about 480 females and 70 males as breeders in the course of each year.

Young adult male hamsters 8 to 12 weeks old and weighing 80 to 110 gm. at the time of initial grafting were employed in all experiments. One or two full-thickness skin grafts about 1 cm. in diameter, from which all fascial tissue ad-

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herent to the graft dermis had been removed, were transplanted to beds prepared in the skin of the host's chest. Care was taken to leave intact the well-vascularized panniculus forming the graft bed. The detailed operative procedures used were previously described by Billingham and Hildemann.[1] The general use of two grafts per recipient facilitated estimation of survival end points, allowed biopsy specimens to be taken, and provided insurance against the occasional loss of a graft through faulty healing. The grafts were initially inspected on the eighth postoperative day, and subsequent inspections were generally made at 3- to 4-day intervals during the first 3 weeks and at weekly intervals thereafter. Animals were kept under observation as long as their grafts showed any degree of survival. Onset and course of rejection, including final survival end points, were assessed on the basis of the outward appearance of the homografts. These assessments were confirmed in many instances by the histological examination of biopsy specimens. The end points of prolonged chronic rejections were by their very nature difficult to determine precisely. The epithelial necrosis, scabbing, or ulceration that signalize the death of acutely rejected grafts did not occur terminally in chronic breakdown. In such grafts complete baldness associated with formation of a dermal collagen pad was ascertained to be the most nearly objective survival end point.

Experimental results. Totals of 55 first-set grafts, 20 second-set grafts, and 11 third-set grafts in duplicate were made and subsequently followed. Only 4 of 55 animals showed typical acute rejection of their first-set grafts in 14 days or less. The onset of such rejection was grossly indicated by hemostasis, inflammation, and epithelial weakness. The remaining 92.7 per cent of grafts gave chronic reactions and survived for periods ranging from 19 to >550 days. These results are detailed in TABLE 1. Three arbitrary classes of chronic rejection are specified in the table in order of increasing survival times: (1) rapid chronic, with survival end points from 19 to 61 days; (2) intermediate chronic, with rejection completed at >78 to >261 days; (3) prolonged chronic, with prolonged survival ranging from 307 to >550 days. Homografts in the chronic classes characteristically healed in well and developed quite normal hair crops within 3 to 4 weeks. The great majority evinced no incompatibility during this period. The first outward sign of the onset of rejection was always a definite, general thinning of the hair crop. It should be noted that the days of onset recorded in the tables were those of the first inspections at which such hair thinning became noticeable. As the hair crop became progressively more sparse, scaly exfoliation of the epithelium (that is, hyperkeratosis) was manifested. In rapid chronic reactions, a leathery graft texture and indistinct collagen pad formation were apparent at this stage. However, in prolonged reactions the graft became progressively thinner and the white collagen pad more conspicuous. These stages of breakdown in progress extended over weeks or even many months before baldness and cicatrization ultimately indicated a zero survival end point (TABLE 3).

The reaction times of first-set homografts summarized in TABLE 1 reveal an important general relationship: the later the time of onset of chronic rejection, the longer the interval between onset and the survival end point. Thus if breakdown began within 19 days after grafting, the grafts were destroyed during the next 14 days or less. If onset occurred between 25 and 43 days, the grafts were destroyed within the following 28 days. However, if rejection began at 120 days or later, the grafts survived for at least 50 and up to 380 or more days thereafter. The interval between onset and survival end point provides a measure of the tempo of the rejection process. This time-interval relationship, however, did show much greater variability in grafts rejected following late onset than in rapid or intermediate rejection. Thus the interval between onset and end point was as short as 112 days or as long as 380 days in the prolonged chronic class of first-set reactions. Although the assessment of late onset times involved a probable subjective error in judgment of about ±7 days, as evidenced by our independent scoring of the grafts, the prolonged survivals observed are nevertheless impressive. It is noteworthy that a few such grafts showed partial loss of hair pigment long before any thinning of the hair crop was evident.

TABLE 1

REACTION TIMES OF FIRST-SET HOMOGRAFTS*

Rejection class	Number of animals	(1) Day of onset of rejection (2) Survival time in days			
		(1)	(2)	(1)	(2)
Acute (14 days or less)	4	<8	8	8	11
		<8	8	11	14
Rapid chronic (19 to 61 days)	14	11	19	28	46
		>16	19	32	46
		15	20	25	53
		11	25	>53	>53†
		18	25	>54	>54†
		18	25	43	57
		19	32	40	61
Intermediate chronic (>78 to >261 days)	16	>78	>78†	117	166
		89	94	81	>172†
		46	110	173	229
		60	110	124	230
		86	113	183	232
		>117	>117†	169	>241†
		110	138	166	243
		>138	>138†	218	>261†
Prolonged chronic (307 to >550 days)	21	>307	>307†	>390	>390‡
		96	311	320	>398†
		194	311	353	495
		201	313	360	517
		159	327	180	>519†
		173	338	323	532
		215	341	166	>545‡
		131	349	327	>545‡
		194	390	474	>550‡
		>390	>390‡	>550	>550‡
		>390	>390‡		

* Grouped in order of increasing survival times.

† Animal died with homografts still viable.

‡ Homografts still viable at last scoring.

To ascertain the immunological nature of chronic reactions, second- and third-set homografts were made at various times after breakdown of the previous sets. The reaction times of repeat-set grafts as well as the time intervals between successive sets are detailed in TABLE 2 in order of increasing first-set survival times. Recipients 1 to 13 in TABLE 2 with first-set survival times ranging from 25 to 349 days all showed accelerated rejection of their second-set grafts, thereby suggesting an immunological basis for the reactions. Although the second-set survival times ranged from 12 to 130 days, the rapidity of the rejections was *not* proportional to the first-set survival times. Thus recipients 3 and 13 both rejected their second-set grafts at 22 days, but the first-set survival times were 110 and 349 days, respectively. Moreover, the degrees of immunity as reflected in the curtailment of survival of repeat-set grafts were not dependent upon the intervals between sets over a very wide range from 48 to 438 days.

TABLE 2

COMPARATIVE REACTION TIMES OF FIRST-, SECOND-, AND THIRD-SET SKIN HOMOGRAFTS*

Recipient No.	First sets		Interval between 1st and 2nd set grafting days	Second sets		Interval between 2nd and 3rd set grafting days	Third sets	
	Day of onset	Survival time in days†		Day of onset	Survival time in days		Day of onset	Survival time in days
1	18	25	48	10	12	49	11	208
2	18	25	245	>11	18	117	>12	20
3	28	46	91	>16	19	—	—	—
4	89	94	364	18	39	—	—	—
5	60	110	140	>14	22	49	11	15
6	86	113	255	53	81	124	27	41
7	110	138	140	>14	22	49	>8	11
8	173	229	246	25	53	115	36	>142§
9	124	230	323	32	53	115	14	29
10	183	232	255	39	53	124	9	12
11	166	243	246	32	88	117	<8	8
12	194	311	330	46	130	—	—	—
13	131	349	438	>14	22	—	—	—
14	>16	19	246	>18	25	117	>12	20
15	11	19	48	>15	20	49	15	23
16	11	25	245	46	221	—	—	—
17	25	53	122	29	123	—	—	—
18	40	61	246	74	>151‡	—	—	—
19	117	166	259	25	272	—	—	—
20	173	338	361	>150	>150§	—	—	—

* Recipients 1 to 13 showed accelerated second-set reactions, whereas 14 to 19 showed either no acceleration or enhanced second-set survival. Among third-set grafts, 6/11 showed further decrease in survival time compared with the second sets.

† In order of increasing survival times for each group (1 to 13 and 14 to 19).

‡ Animal died with homografts still viable.

§ Homografts still viable at last scoring.

Second-set grafts on recipients 14 through 19 gave surprising results. With the possible exception of No. 15, these grafts all survived for longer periods than the first sets, in most instances very much longer. This enhancement of re-

TABLE 3

SYNOPSIS OF PATHOLOGICAL FEATURES OF CHRONIC SKIN HOMOGRAFT REJECTION IN THE SYRIAN HAMSTER

Type of chronic rejection		Phase of rejection		
		Early: 100 minus to 90% survival score	Middle: 90% to trace survival score	Late: zero and postzero survival score
Rapid (2 months or less total survival)	*Gross features*	Slight thinning of hair crop. Occasional epithelial scaliness.	Rapid loss of hair. Scaly with occasional matted hair stumps. Leathery graft texture.	Hair very sparse or absent. Cicatrization. Contraction (irregular). White collagen pad conspicuous.
	Histological features Superficial epidermis	Early but incomplete necrobiosis of granular layer, mild vacuolar degeneration of basal layer.	Intact except for mild vacuolar degeneration of basal layer. Thickened and hyperkeratotic, esp. in 2nd and 3rd sets.	Intact. Slightly thickened and hyperkeratotic in 2nd and 3rd sets.
	Epidermal appendages	Surrounded by intense round cell infiltrate.	Slightly to much reduced in number. Surrounded by moderate round cell infiltrate.	Virtually absent.
	Granulomatous reaction	Moderate in deep dermis. Stubs of hair surrounded by foreign body giant cells.	Moderate in deep dermis.	Moderate to intense in deep dermis.
	Dermal connective tissue	Beginning hyalinization. Intense subepidermal round cell infiltrate.	Continued hyalinization. Moderate subepidermal round cell infiltrate.	Distinct collagen pad.
	Inflammatory cells*			
	Plasma cells	++	+ to ++	few
	Round cells	++ to +++	++	irregular
	Neutrophils	few	few	few or none
	Mast cells	few	few	few

	Gross features	Slight thinning of hair crop.	Gradual loss of hair. Pale shiny graft.	Hair very sparse or absent. White collagen pad conspicuous.
	Histological features Superficial epidermis	Generally intact. Often thickened and hyperkeratotic.	Intact, occasional thickened and hyperkeratotic. Occasional vacuolar degeneration of basal layer.	Intact, occasional hyperkeratotic.
Intermediate and prolonged (2 to 16 months total survival)	Epidermal appendages	Surrounded by moderate round cell infiltrate.	Moderately reduced in number. Moderate round cell infiltrate.	Virtually absent.
	Granulomatous reaction	Mild to moderate but inconstant.	Mild to moderate but inconstant.	Minimal or absent.
	Dermal connective tissue	Beginning hyalinization.	Continued hyalinization.	Distinct collagen pad.
	Inflammatory cells*			
	Plasma cells	++	few	few
	Round cells	+ to +++	+ to ++	+
	Neutrophils	none	none	none
	Mast cells	+	+ to ++	+

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* The relative abundance of such cells is indicated as ranging from none to +++ within each cell-type category.

peat-graft survival, though not unusual in tumor homotransplantation, appears inexplicable in the present situation in view of the diminution of repeat-graft survival times found in the majority of animals under similar conditions.

Considering the 11 third-set grafts as a group, nearly all possible outcomes were realized. Heightened immunity when compared with the second sets, that is, further decreased survival time, was observed in 6 of 11 recipients (Nos. 5, 6, 7, 9, 10, 11). Greatly enhanced survival occurred in two hamsters (Nos. 1 and 8) which had previously destroyed their second-set grafts after accelerated reactions. Finally, slight diminution (No. 14) and no further diminution or slight enhancement (Nos. 2 and 15) of third-set survival times as compared with the second sets were observed. The conspicuous differences in survival times and onset of rejection times show no apparent relationship to either the second-set survival end points or to the interval between second- and third-set grafting. Although repeat-set grafting led to increased transplantation immunity for the most part, the accelerated rejections nevertheless remained chronic in nature, with only a few such grafts succumbing with typical acute reactions.

Pathological features. The histopathological picture during the course of chronic rejection was reconstructed from study of skin biopsy sections of 16 first-set, 8 second-set, and 3 third-set grafts. Representative biopsies were obtained at early, middle, and late phases of graft breakdown, as well as 1 to several weeks postrejection. These phases of rejection, originally classified according to distinctive gross features, also revealed certain characteristic histological features. The histological pattern in turn depended more on whether the chronic rejection was rapid (within 2 months or less) or prolonged (2 to 18 months), than whether the grafts were first, second, or third set. The comparative pathological findings are synoptically presented in TABLE 3.

In "rapid chronic rejections" (see TABLE 3), corresponding to the rapid chronic class of TABLE 1, the initial changes (100 minus to 90 per cent survival score) consisted of focal vacuolar degeneration and partial disappearance of the basal layer of the epidermis, and considerable decrease in number of epidermal cell nuclei per unit area (FIGURE 1*a*). In no instance, however, could the epidermis be regarded as "necrotic." The epidermal appendages during the early phase were surrounded and partly obliterated by an intense chronic inflammatory reaction predominantly associated with lymphocytes and reticuloendothelial cells (FIGURE 1*b*). Similar cells appeared subepidermally in considerable numbers (FIGURE 1*a*), and were also scattered through the deep dermis. Numerous plasma cells singly and in clusters were also noted in the deep dermis (FIGURE 2*a*) having both a diffuse and perivascular distribution. During early and middle phases a granulomatous reaction, chiefly involving hair shafts and hair follicle roots, was evident in the dermis and subdermal fat (FIGURE 2*b*). It was characterized by accumulation of epithelioid cells, foreign-body giant cells, and Langhans' giant cells into discrete granulomata. Occasionally the stubs of broken hair shafts were discernible in the centers of these granulomata (FIGURE 2*b*). Beginning hyaline degeneration of dermal collagen was also observed at this time.

Inflammatory cells gradually diminished in number during the middle phase of rapid chronic rejection (90 per cent to trace survival score), with a steep de-

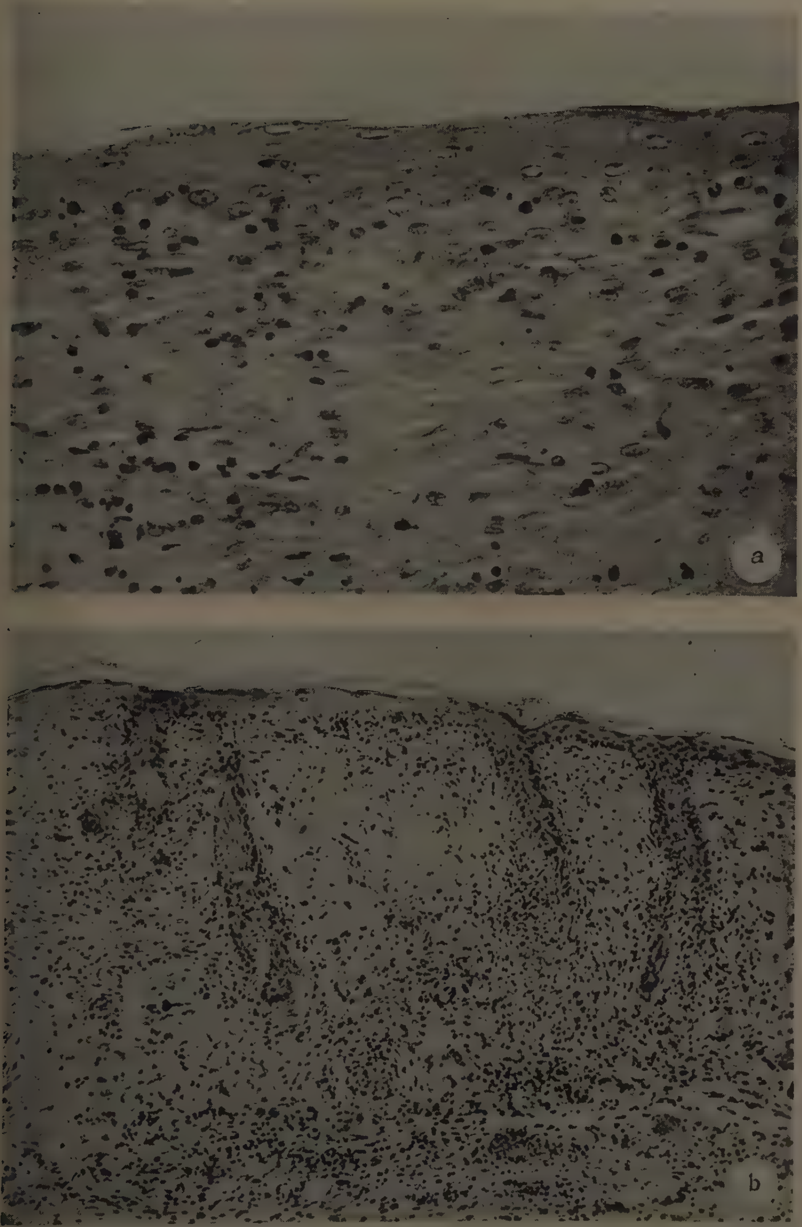

FIGURE 1. Early phase of first-set-homograft rejection. (*a*) Note partial disappearance f the basal layer of the epidermis, decrease in number of epidermal cell nuclei per unit area, nd a moderate subepidermal chronic inflammatory response. (*b*) The beginning of obliteraion of epidermal appendages by chronic inflammation. A moderate to intense chronic inammatory reaction in the dermis is also seen.

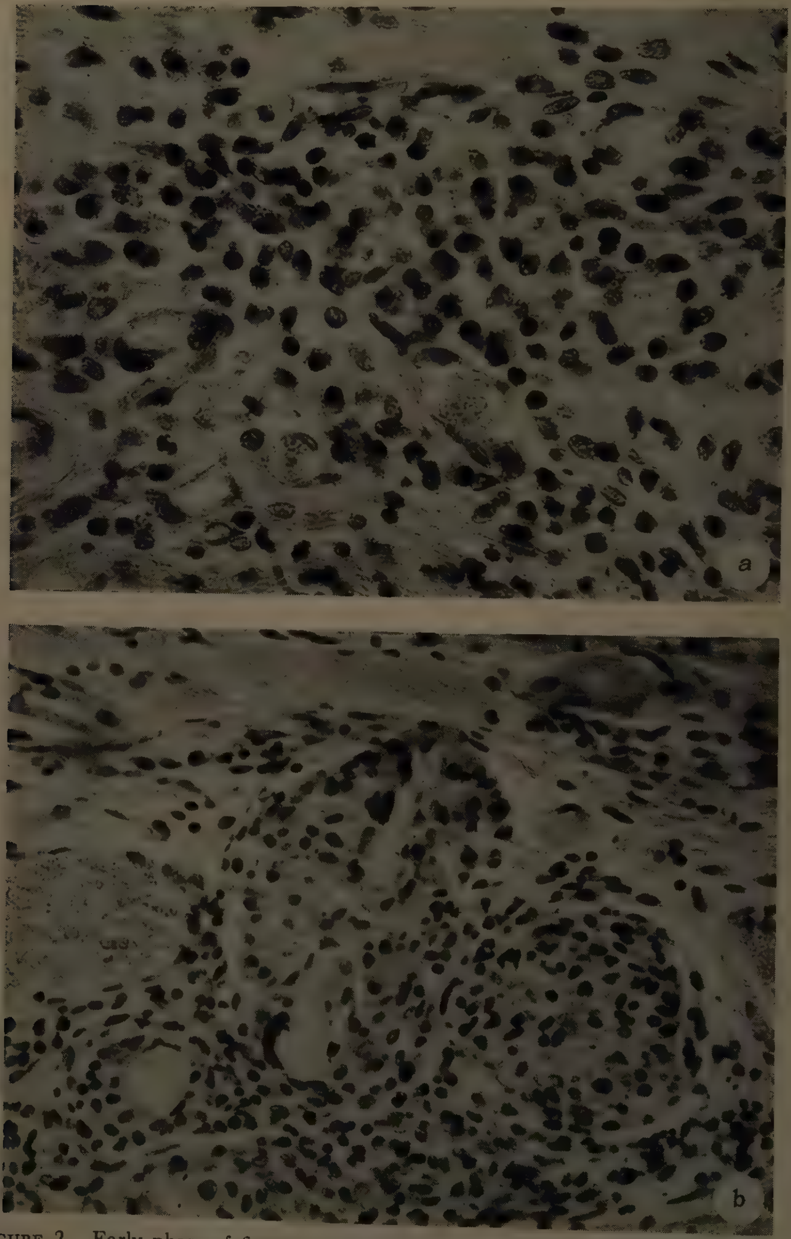

FIGURE 2. Early phase of first-set homograft rejection. (*a*) Large numbers of plasma cells are present in the deep dermis. (*b*) A granulomatous reaction in the dermis is shown. The degenerating hair shaft surrounded by giant cells (running from 7 to 1 o'clock through center of photograph) is clearly evident.

cline in the severity of the inflammatory process at the time of the zero end point. While the epidermis appeared relatively intact during the middle phase, it was sometimes thickened and hyperkeratotic, especially in second- and third-set grafts. Epidermal appendages, although reduced in number, remained surrounded by inflammatory cells throughout the middle phase and were not entirely obliterated until very late. Concurrently, the so-called collagen pad of resistant connective tissue developed as a broad hyaline band in the mid-dermis. Biopsies at the zero end point or shortly thereafter showed a still intact epidermis with few or no epidermal appendages, a distinct collagen pad (FIGURE 3*a*), and minimal inflammation. The granulomatous reaction was quite variable at this stage and could be absent or very intense (FIGURE 3*b*). No exact graft survival end point could be determined histologically.

In "prolonged chronic rejection" (TABLE 3), corresponding to homografts in the intermediate and late chronic classes of TABLE 1, the reactions described above were generally milder and extended over longer periods of time. However, during the early phase the epidermis was often conspicuously hyperkeratotic and thickened (FIGURE 4*a*), but rarely showed necrobiotic alterations. Although plasma cells were numerous early in the course of rejection, few were present thereafter. Large numbers of typical mast cells were noted (FIGURE 4*b*), especially during the middle phase of prolonged reactions, in contrast with the paucity or absence of such cells during all phases of rapid chronic rejection.

Accelerated breakdown of repeat-set grafts was reflected histologically by intense chronic or even subacute inflammation and a pronounced giant-cell granulomatous reaction. Nevertheless, the pattern of reactions was similar to that seen in first-set grafts, with comparable onset and survival times.

Discussion

A primary immunological basis for chronic homograft rejection is suggested by the fact that most repeat-set grafts succumbed following accelerated reactions. However, the incompatibility developed slowly and generally remained weak, as evidenced by the histological sections at successive stages of breakdown in progress. It is not unlikely that the 6 of 11 third-set grafts that showed clearly accelerated breakdown in comparison with the second sets reflect an anamnestic response, although the over-all results are equivocal in this respect. The enhanced survival of a number of repeat-set grafts following a more rapid destruction of the previous set resembles the "enhancement" of homograft survival provoked in mice,[11,12] rats,[13,14] and rabbits[15,16] that were pretreated with certain donor cell preparations. Nevertheless, the present results are particularly puzzling in this connection, because the majority of animals rejected their repeat-set grafts more rapidly under almost identical experimental conditions. However, considerable isoantigenic heterogeneity within the colony (*see below*) might in some way account for the different results. Considering the 19 hamsters that rejected both first- and second-set grafts as a whole, one can statistically evaluate certain possibilities. Thus it is conceivable that the curtailed or enhanced survival times of second-set grafts might be influenced by the age of the animals as a consequence of the long time intervals involved. However, a chi-square test based on such a two-way classification[17]

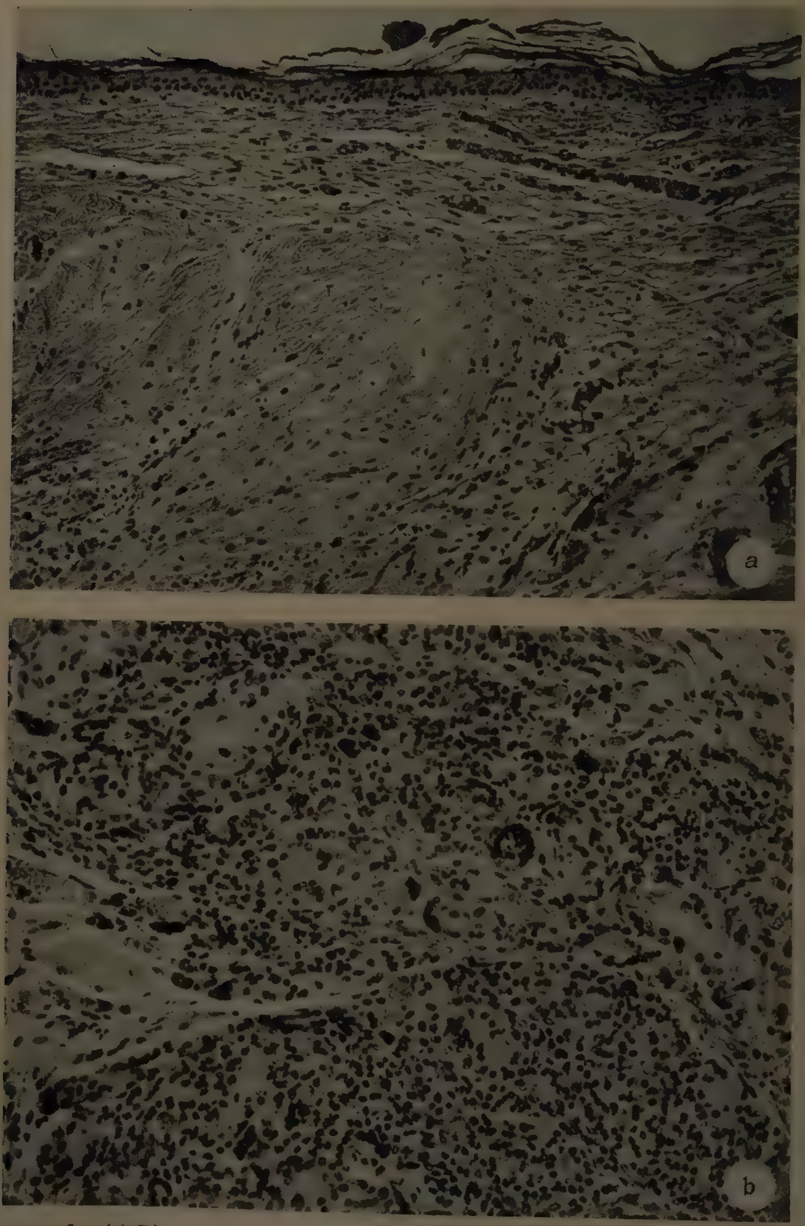

FIGURE 3. (*a*) Biopsy at zero end point of rapid, chronic, first-set homograft rejection. A well-outlined collagen pad of residual graft connective tissue is present in the mid-dermis. (*b*) Biopsy at 10 days postzero end point of rapid, chronic, first-set homograft rejection. Marked granulomatous reaction in the deep dermis is noted.

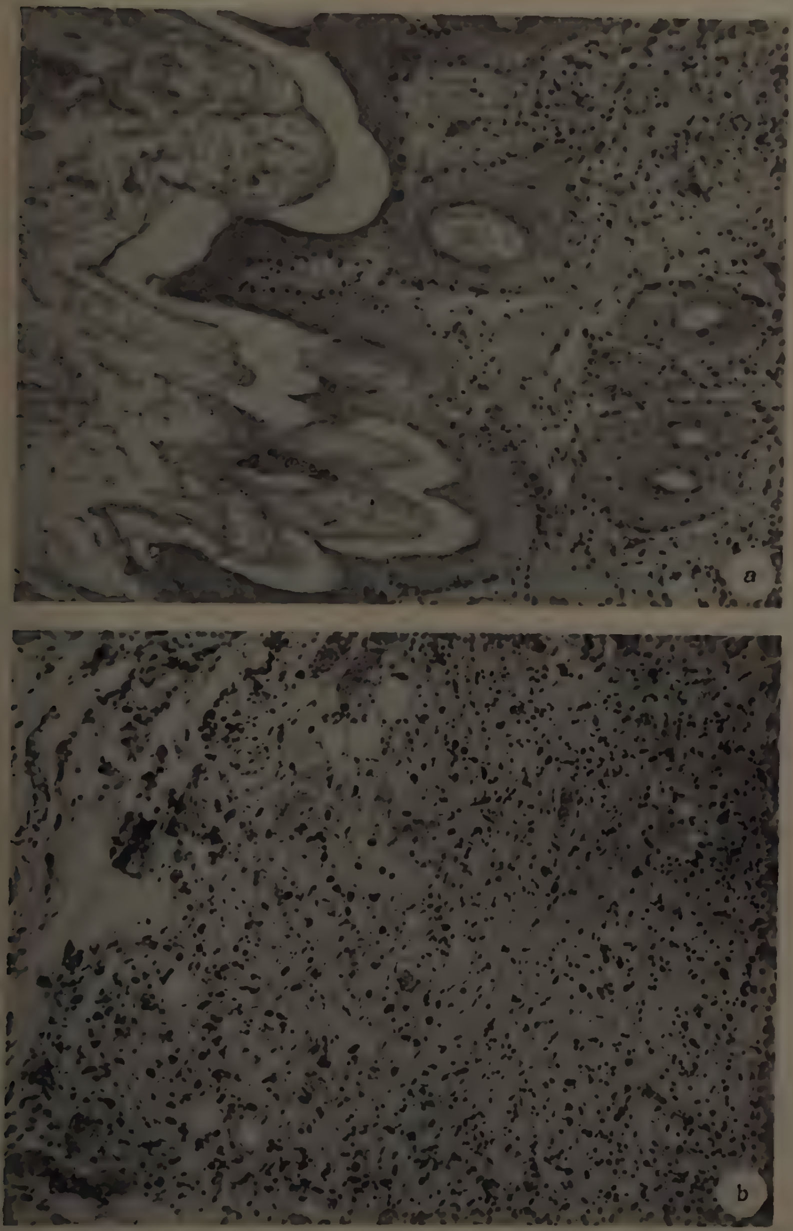

FIGURE 4. (*a*) Early phase of prolonged chronic homograft rejection The epidermis is greatly thickened and covered by a hyperkeratotic scale. (*b*) Middle phase of prolonged hronic homograft rejection. Large numbers of mast cells (intensely dark cells in the photograph) are scattered throughout the dermis.

supports the supposition that the age of the hamsters and graft survival times are quite independent. Although the strikingly curtailed survival times of second-set grafts observed on 13 of 19 animals point to immune rejection, a nonparametric sign test[17] comparing the 13 curtailed to 6 enhanced survivals reveals a 25 per cent probability that this distribution as such could represent an equal chance of either outcome. In other words, the supposition that chronic homograft rejection is mediated primarily by immune reactions cannot be proved statistically with these results. This may be a consequence of dealing with two distinct phenomena: (1) an anamnestic-type response and (2) an enhancement response, whose effects cancel each other statistically.

It is improbable that any single type of immune reaction per se could account for the variable patterns of chronic skin homograft rejection observed in these hamsters. Rather, the sequence of events during the course of graft breakdown and replacement indicates a continuing "struggle" between graft and host, with graft cell proliferation partly replacing those cells destroyed by the host. In addition, the different rates of breakdown of various graft components (basal layer of epidermis, granular layer of epidermis, collagen) may reflect a differential susceptibility of certain tissues to the effects of weak immune reactions. Breakdown of graft cells and invasive replacement by host cells must take place more or less concomitantly in prolonged rejections, because ulceration of the graft surface is not seen. Moreover, extensive contraction of postzero grafts occurred only after acute and rapid chronic reactions, irrespective of the graft set involved. It is likely that the host epidermis subtly overgrows and gradually replaces the graft epidermis. The primary histological manifestation of the homograft reaction appeared to be the early influx of plasma cells and lymphoreticular cells, with persistence of the latter throughout the breakdown period. The earlier the onset and the shorter the total reaction period, the more intense was the lymphoreticular cell response. Although the granulomatous reaction was a conspicuous feature in many grafts, we believe that it represented a response to poorly digestible irritant substances such as hair shafts and sebaceous material. Indeed, the reaction resembled that seen about ruptured sebaceous cysts and pilonidal cysts in man. The observed resistance of the collagen matrix to destruction in otherwise rejected homografts has also been seen in the joined ears of incompatible rabbits. The ears remained firmly united by poorly cellular collagenous tissue long after cross-circulation had ceased.[18]

Although some homografts on the hamsters retained their integrity as long as the optimum life expectancy of the animal (about 18 months), very few qualified as permanently successful grafts. In other words, few homografts were still fully viable at the age when the animals became senile. It follows from this finding that prolonged homograft compatibility is not necessarily a valid criterion of complete genetic or antigenic homogeneity. Nevertheless, in species such as the mouse, in which a difference in any one of at least 15 independent histocompatibility loci is presumed to cause more or less vigorous homograft rejection,[19] prolonged homograft acceptance is probably as satisfactory a measure of genetic uniformity as any other.

That relative weakness of histocompatibility barriers is not a phenomenon peculiar to Syrian hamsters is indicated by a variety of recent studies. For example, Mariani *et al.*[20] showed that not only newborn but adult female mice may be made tolerant of male skin isografts if injected with male splenic cells, although the successful test grafts were observed for only 4 to 12 months. Even more surprising in this connection is the report[21] that normal, adult Z(C3H) mice, connected in parabiosis with isogenic mice tolerant of Ce strain tissue, fail to reject homografts of Ce skin, and that such unresponsiveness persists following disruption of parabiotic union. In a large colony of noninbred Wistar rats, Billingham and Silvers[7] observed that, while the majority of animals promptly rejected skin homografts, a considerable number of grafts survived for 21 to 50 days or much longer. The investigation of the role of strong and weak histocompatibility gene differences in the rejection of mouse skin homografts already cited[3] is most enlightening in this regard. Recently Berrian and Jacobs[22] have analyzed the diversity of transplantation antigens in 4 different strains of mice. Their findings elegantly illustrate the point that the early rejection of grafts seems to depend on immunity against relatively few strong (H-2) antigens, and that the effects of weak antigens are usually obscured by the presence of stronger ones. While Converse and Rapaport[23] ascertained that skin homografts exchanged between unrelated humans survive for only 2 weeks or less, Peer[24] found that prolonged graft survivals occurred reciprocally when skin was exchanged between mothers and their male or female infants. The latter provocative finding certainly deserves further investigation.

Woodruff[25] has recently reappraised the evidence bearing on his adaptation hypothesis, according to which some types of homografts when once established become progressively less vulnerable to immunological attack. The histopathological changes during prolonged homograft survival in hamsters, however, indicate that gradual destruction and replacement of graft cells by host cells always take place. Nevertheless, we did observe a few homografts that suffered an early setback, with scaliness and thinning of hair crops, but then showed a gradual return to apparently full viability before late, inexorable rejection occurred. The possibility cannot at present be ruled out that intrinsic changes in graft or host cells leading to less incompatibility might be induced under certain circumstances.

So-called noninbred animals produced by "random" matings in a relatively small colony might be expected to share many isoantigens in common as a result of inbreeding and genetic drift. In the present experiments, however, prolonged homograft acceptance cannot be explained on this basis, since the hamsters employed were the product of ten years of strict outbreeding within a large, closed colony. The genetic basis for the apparent lack of the usual antigenic disparity within the various random-bred colonies of Syrian hamsters investigated so far is unknown. Unusual genetic stability is an unlikely explanation, because many other types of mutants have been identified in recent years.[26,27] Moreover, serologic experiments now in progress have revealed individual differences in red cell antigens within and between several closed colonies of Syrian hamsters (W. H. Hildemann, unpublished data). Eventually we hope to be able to correlate the histocompatibility antigens with the individual isoantigens detected serologically. Closely inbred lines of the histoincompatible colonies previously investigated[1] are being produced for this purpose. The genetics of transplantation in the Syrian hamster may conceivably turn out to be less complex and therefore more amenable to general analysis than is the case in the mouse, where the numerous H-2 alleles play a pre-eminent role.[28]

Summary

Skin homografts in adult Syrian hamsters from a closed but continuously outbred colony displayed highly variable rates of rejection, reflected both in onset times and total survival times. Fifty-one of 55 first-set homografts showed chronic reactions, with survival times ranging from 19 to >550 days. Indeed, the majority of such grafts remained fully viable for many weeks or months, although only a few homografts qualified as permanently successful by remaining intact for the life span of the animal (about 18 months in captivity).

The chronic reactions revealed an important general relationship: the later the time of onset, the longer the interval between onset and the survival end point. Moreover, a measure of the rate of rejection was provided by the interval between onset and survival end point. This interval varied from 112 to 380 days in the prolonged chronic class of first-set reactions.

The conspicuously accelerated destruction of most repeat-set grafts suggests a primary immune basis for chronic homograft rejection in this species. However, a number of second- and third-set grafts showed greatly enhanced survival under similar conditions. Although long time intervals were involved in these experiments, it was determined statistically that the observed graft survival times were independent of the age of the animals. Considerable isoantigenic heterogeneity within the colony might in some unknown way account for the different results.

It is improbable that any single type of immune reaction per se could be responsible for the variable patterns of chronic skin homograft rejection found. The sequence of events during chronic reactions indicates a protracted struggle between graft and host, with breakdown of graft cells and invasive replacement by host cells taking place gradually and more or less concomitantly. Histopathological changes in first-, second-, and third-set grafts at various phases of breakdown are described. The primary histological manifestation of the homograft reaction was the early influx of plasma cells and lymphoreticular cells, with persistence of the latter throughout the breakdown period. The earlier the onset and the shorter the total reaction period, the more intense was the lymphoreticular cell response.

This study supports the hypothesis that many 'weak' but relatively few 'strong' histocompatibility antigens are generally present in Syrian hamsters. Neither inbreeding nor infrequency of mutation can be invoked to explain the observed lack of the marked histoincompatibility that is found in other species.

Acknowledgment

It is a pleasure to acknowledge the technical assistance of Margaret Morlino during this prolonged investigation.

References

1. Billingham, R. E. & W. H. Hildemann. 1958. Studies on the immunologic responses of hamsters to skin homografts. Proc. Roy. Soc. London. **B148:** 216–233.

2. Adams, R. A., D. I. Patt & B. R. Lutz. 1956. Long term persistence of skin homografts in untreated hamsters. Transplantation Bull. **3:** 41–42.
3. Counce, S., P. Smith, R. Barth & G. D. Snell. 1956. Strong and weak histocompatibility gene differences in mice and their role in the rejection of homografts of tumors and skin. Ann. Surg. **144:** 198–204.
4. Billingham, R. E. & W. K. Silvers. 1958. Induction of tolerance of skin isografts from male donors in female mice. Science. **128:** 780–781.
5. Zaalberg, O. B. 1959. An analysis of the Eichwald-Silmser effect. Congr. Coll. Univ. Liége. **12:** 306–312.
6. Medawar, P. B. & M. F. A. Woodruff. 1958. The induction of tolerance by skin homografts on newborn rats. Immunology. **1:** 27–35.
7. Billingham, R. E. & W. K. Silvers. 1959. The induction of tolerance of skin homografts in rats with pooled cells from multiple donors. J. Immunol. **83:** 667–679.
8. Billingham, R. E. & L. Brent. 1959. Quantitative studies of tissue transplantation immunity. IV. Induction of tolerance in newborn mice and studies on the phenomenon of runt disease. Proc. Roy. Soc. London. **B242:** 439–477.
9. Cannon, J. A., P. Terasaki & W. P. Longmire, Jr. 1957. Study of factors influencing induced tolerance to skin homografts in the chicken. Ann. Surg. **146:** 278–284.
10. Hildemann, W. H. & R. Haas. 1959. Homotransplantation immunity and tolerance in the bullfrog. J. Immunol. **83:** 478–485.
11. Kaliss, N. 1959. Immunological enhancement of tumor homografts in mice. Cancer Research. **18:** 992–1003.
12. Billingham, R. E., L. Brent & P. B. Medawar. 1956. 'Enhancement' in normal homografts with a note on its possible mechanism. Transplantation Bull. **3:** 84–88.
13. Parkes, A. S. 1956. Attenuation of host reaction to ovarian homografts. Nature. **178:** 1228.
14. Parkes, A. S. 1958. Enhancement of the survival of interstrain ovarian homografts in rats. Transplantation Bull. **5:** 45–47.
15. Billingham, R. E. & E. M. Sparrow. 1955. The effect of prior intravenous injections of dissociated epidermal cells and blood on the survival of skin homografts in rabbits. J. Embryol. Exptl. Morphol. **3:** 265–285.
16. Albert, F. & G. Lejeune. 1959. Prolonged survival of skin homografts. Congr. Coll. Univ. Liége. **12:** 109–126.
17. Dixon, W. J. & F. J. Massey. 1957. Introduction to Statistical Analysis. : 1–488. McGraw-Hill. New York, N. Y.
18. Andresen, R. H., C. W. Monroe & G. M. Hass. 1954. Cross circulation and tissue reactions in parabiosis. A.M.A. Arch. Pathol. **58:** 455–474.
19. Barnes, A. D. & P. L. Krohn. 1957. The estimation of the number of histocompatibility genes controlling the successful transplantation of normal skin in mice. Proc. Roy. Soc. London. **B146:** 505–526.
20. Mariani, T., C. Martinez, J. M. Smith & R. A. Good. 1959. Induction of immunological tolerance to male skin isografts in female mice subsequent to neonatal period. Proc. Soc. Exptl. Biol. Med. **101:** 596–599.
21. Martinez, C., J. M. Smith, F. Shapiro & R. A. Good. 1959. Transfer of acquired immunological tolerance of skin homografts in mice joined in parabiosis. Proc. Soc. Exptl. Biol. Med. **102:** 413–417.
22. Berrian, J. H. & R. L. Jacobs. 1959. Diversity of transplantation antigens in the mouse. Congr. Coll. Univ. Liége. **12:** 131–145.
23. Converse, J. M. & F. T. Rapaport. 1956. The vascularization of skin autografts and homografts. An experimental study in man. Ann. Surg. **143:** 306–315.
24. Peer, L. A. 1958. Behavior of skin grafts exchanged between parents and offspring. Ann. N. Y. Acad. Sci. **73**(3): 584–589.
25. Woodruff, M. 1959. Evidence of adaptation in homografts of normal tissue. Congr. Coll. Univ. Liége. **12:** 83–90.
26. Robinson, R. 1958. Genetic studies of the Syrian hamster. I. The mutant genes cream, ruby-eye and piebald. J. Genetics. **56:** 1–18.
27. Robinson, R. 1959. Genetic studies of the Syrian hamster. II. Partial albinism. Heredity. **13:** 165–177.
28. Gorer, P. A. 1959. Some recent data on the H-2 system of mice. Congr. Coll. Univ. Liége. **12:** 25–30.

Discussion of the Paper

Donald L. Ballantyne, Jr. (*New York University School of Medicine, New York, N. Y.*): My comments will probably comprise a very brief description of studies made by Converse, Rogers, and myself, confirming in the laboratory rat the findings of Hildemann in the hamster: namely, the existence, grossly and histologically, of the "chronic rejection" phenomenon. In our laboratory we have performed 366 reciprocal homotransplantations of skin between CFN rats specifically bred for their heterogeneity but closely related, and also between "open-stock" closely related rats. We have already reported these studies in so far as the MST or "median survival-time" aspect of them is concerned at the May 1959 meeting of the American Association of Plastic Surgeons in Boston and at the July 1959 meeting of the Second International Congress of Plastic Surgery held in London, England.

Our histological observations show that a similar chronic rejection occurs in closely related CFN rats (for example, mothers and daughters) who receive each other's reciprocally transplanted skin homografts in a varying percentage of the total animals grafted. If, among this total number of animals, the homograft does not undergo an acute rejection, it may undergo one of two possible reactions: (1) a chronic rejection phenomenon that we have termed the "scar reaction," or (2) the reaction of "prolonged survival." In the "scar reaction," the graft appears relatively normal in its early stages, but seems to shrink from its edges over a period of 43 to 76 days until it reaches its end point, namely, a fine linear scar. We found it interesting that, of 366 homografts, 95 showed the chronic rejection in a scar pattern that took place from 43 to 76 postoperative days. In 21 of the 366 grafts, we had a prolonged survival, the longest of which was 204 days postoperatively, when these animals were then sacrificed for histological study. It would be interesting to know how long the latter grafts would have survived and whether their end points would also be ultimate rejections as demonstrated in the very prolonged chronic rejections described by Hildemann.

Histologically, the scar reaction in rats is typified by a mild lymphocytic response occurring primarily at the dermal epidermal junction of the graft, at the junction of the graft itself with the host tissue and, specifically, around the blood vessels of the graft and the junction of the graft blood vessels with the host blood vessels.

In "prolonged survival," that is, in those grafts surviving for as long as a period of 204 days, the only way we could differentiate between the graft that still may be undergoing some degree of chronic rejection was by a slight thickening of the collagenous fibers of the graft at the host-graft junction and, with the exception of scattered lymphocytes throughout the graft dermis, it was very difficult to state categorically that this graft was undergoing any rejection at the time it was examined (for example, 204 days). Grossly, the graft was still identifiable by the 180-degree reversal of its hair-growth pattern.

I think, therefore, that we now have on record another animal, the laboratory CFN rat, in which the chronic rejection phenomena so well described by Hildemann in hamsters have been conclusively demonstrated in our laboratory in more than 366 rats specifically bred for their heterogeneity.

Blair O. Rogers: One point of clinical significance that these chronic rejection phenomena may have at present, to be a little pessimistic, would be to serve as a caution to those clinicians who might wish to transplant homografts of other tissues (for example, kidney) from a parent or other relative to an offspring or relative. They should be reminded that perhaps their period of postoperative observation of these organ or tissue homografts in the human should be an increasingly longer one. An organ or any other type of homografted tissue conceivably could be undergoing this chronic type of rejection over a very long period of time, for example, one year or more, and this rejection might not be apparent if observed for only a short postoperative period. This might probably be the state of affairs in the current behavior of the kidney homotransplant obtained from a nonidentical twin donor in the recent case described by Joseph E. Murray and his associates. On the other hand, those few instances of extremely long survival of organ and tissue homografts in some experimental animals and a few humans might require a different explanation, such as the existence of a state of "partial tolerance," to account for their apparent acceptance by the host. Nevertheless, the use of a reciprocally transplanted skin homograft in humans by those who wish to determine which most likely candidate of a close genetic relationship, for example, a mother, is to be used as donor of a subsequently transplanted organ, for example, a kidney, is a procedure that is not entirely lacking in possible unfavorable complications. Since skin and kidney share antigens in common, it is not entirely impossible that at some time in the future some surgeon will use this "skin homograft test," only to find that conceivably it might shorten the ultimate length of survival of a subsequently transplanted organ because of its initiation of a "second-set" response, no matter how chronic that second-set response might be. Other work in our laboratory suggests that the second- and third-set responses still take place although slowly, with the usual reduction in survival time when subsequent second- and third-set skin homografts are transplanted to the same recipient rat, even though the first-set survival of skin reciprocally transplanted between mothers and their daughters, for example, was extremely prolonged before it was chronically rejected in a "scar reaction" several months after surgery. The second-set survival time in these cases was considerably shortened in comparison to the first set in some animals, even though it, too, was not an "acute" reaction, but was drawn out over several to many weeks in duration before it also had its end point in a scar.

This word of caution is not to discourage the use of skin homografts clinically to test various genetic relationships. Certainly when Allen and I were the first to report the use of this procedure in *Science* in 1955 to distinguish monozygotic from dizygotic human twins, we did so without thinking that the "second-set" reaction would become a problem to those engaged in clinically necessary organ transplantation. Within the past 5 years, however, some clinicians engaged in organ and tissue transplantation have taken up the "skin homograft test" procedure as a help to their choice of an ideal organ donor. There are those who would argue that the continuing survival of a homografted kidney in the therapeutically irradiated fraternal or dizygotic twin described by Murray and his associates, following the chronic rejection of the normal twin's skin homograft after its apparent survival for 6 months, negates this word of caution. It should be remembered in retrospect, however, that the skin homograft from the normal twin was applied prior to X irradiation of the recipient twin, in whom a uremic state could easily account for the graft's initial prolonged survival. In addition, the factors of uremia, irradiation, the time of application of the kidney, and a consanguinity of 26 identical blood groups, could and must be taken into consideration when one tries to evaluate the quantitatively longer survival of the kidney up to the present time. Only a long-term, close follow-up of the survival time of this kidney may or may not demonstrate whether it too suffers the fate of a long drawn-out chronic rejection. Let us hope, of course, for the patient's sake, that this does not take place. If it does, however, we still, unfortunately, shall not know whether in this particular case the ultimate rejection is due to a "chronic second-set response" initiated by the skin homograft test procedure or merely due to the basic genetic incompatibility of their being dizygotic, not monozygotic, twins.

HELENE WALLACE TOOLAN (*Memorial Center for Cancer and Allied Diseases*): I was very much interested in the work reported by Hildemann. Rogers asked me to comment on some work we did with rabbits several years ago in which we emphasized the role of the ground substance. It has seemed to me that, rather than look for a 1:1 ratio between lymphocytes or any particular cell and the graft rejection, we should consider the possibility of a series of events.

In a group of rabbits that we grafted and treated with cortisone, we were able to keep the skin homografts alive as long as we wished, provided the dosage of cortisone was correct. They were kept alive, let us say, for as long as six months. At the end of that time, we discontinued the cortisone and gave the rabbits ACTH. Since the rabbit puts out "B" as a steroid rather than "E" and "F," we were able to study a sort of "slow-motion" rejection of that graft over a subsequent period of about a month following cessation of cortisone administrations.

During this month, the first thing that we noticed, before anything else happened, was a hyalinization of the ground substance. This occurred immediately after the cortisone was discontinued, and it lasted about ten days.

It was only after this had been in evidence for some time that we saw an influx of inflammatory cells: first, polymorphs and, eventually, the lymphocytes. We also, in certain instances, saw mast cells, just as Hildemann has described. I was particularly interested in his mention of them because it seems to me that we might very well have a situation in which the mast cells might be supplying some substance to that ground substance in which it is weak, perhaps one of the sulfates or some other substance that has been presumed by some investigators to have been supplied by the mast cells. Unfortunately, when one has the more usual rapid rejection of homografts, one sees only the overwhelming cellular response and may not see these other changes that might have preceded it.

I think that this might draw attention again to the other histological phenomena that take place long before we see the cellular infiltration.

CHASE: In the finding of one species—the hamster—which exhibits such long latent periods before homograft rejection and now in another species also possessing a rather prolonged period of tolerance, some questions may be raised as to why this is possible and whether the events of early or of late rejection might be foretold. I hark back once again to the rather remarkable finding of Saenz[1] that hamsters infected with BCG very seldom gave the expected granulomatous responses to this type of waxy material that we are accustomed to find around such deposited and scarcely multiplying material in most species. Saenz found, I recall, seven animals of forty-four that were capable of showing granulomas. Can discrepancies of this sort be hereditary in nature and exist in outbred, even though closed, stocks in which one tries to avoid strict consanguinity? Can the difference between one individual and another be detected as a relative readiness to form granulomatous responses to mycobacterial cells? If delayed rejection is associated with the delayed type of allergic response, then an exploration could be undertaken, I believe, as to whether the capacity to form granulomas promptly and well correlates with the ability to reject tissue earlier and more completely in a short period of time. One thing that might possibly be used either to predict the outcome in advance or to ascertain after the event whether the principle were to hold might be to inject dead tubercle bacilli suspended in paraffin oil and to study the kind of cellular response that differentiates one individual from another.

R. A. ADAMS (*Childrens Hospital Medical Center, Boston, Mass.*): In the absence of precise genetic information in hamsters, that is, such as would be obtained from the use of adequately inbred strains, all results with skin homografting in hamsters thus far reported are also explainable on the basis of a randomly distributed ability of the hamster to respond to tissue antigens. This explanation received some support from published work by Handler and, latterly, by Handler and myself in which strain-specific mouse lymphomas (P1534) and a number of other tumors were transplanted to normal hamsters without conditioning. These lymphomas which will kill only the mouse strain of origin proved to grow progressively in normal hamsters and were maintained by serial passage for well over two years, retaining their genetic identity as proved by retansplantation to a series of mice.

W. K. SILVERS (*Wistar Institute, Philadelphia, Pa.*): Is this in the cheek pouch?

ADAMS: This is in the cheek pouch.

SILVERS: I think that the apparently good results of Adams are cheek pouch-specific, since this site is, for some reason, a privileged one, where even heterografts are known to persist without suffering the expected consequences. I think that if Adams had tried to put these transplants anywhere else in the hamster, he probably would not have obtained the same results.

It appears that the Syrian hamster, as shown by the nice work of Billingham and Hildemann, can reject a skin homograft as promptly as other rodents. It appears that the peculiarity in the hamster is that there are very few histocompatibility mutants, so that most populations are almost uniform with respect to these factors. A study in progress with Billingham has been concerned with establishing how many genes are involved in graft acceptance or rejection between two closed colonies of hamsters that reject each other's skin grafts in a matter of ten to twelve days. Skin homografts are almost always accepted within each colony, so that these strains, although not isogenic, are apparently homogeneous with respect to their histocompatibility genes. An F_2 test on these two colonies of animals (challenging the F_2 with parental strain grafts) has revealed that there is only one strong histocompatibility gene difference between these two strains and, probably, two weak differences. There may be other weak histocompatibility factors that we have not been able to detect with skin grafts.

HILDEMANN: I might also add to our earlier published findings that, having now inbred two incompatible lines through the F-7 generation, we have recently done much cross-grafting between these lines. Without exception, we find that the recipients vigorously reject the graft and, to my mind, this refutes the notion that the hamster for some unknown reason is incapable of responding vigorously. If an adequate stimulus or the right graft is made, a hamster can destroy it just as a mouse or a rabbit or any other animal might do so.

May I ask my colleague, R. L. Walford, if he will amplify briefly the histopathological observations with respect to the granulomatous reaction in answer to Chase's comment. I had the feeling in looking at a number of our grafts that these reactions are quite conspicuous.

R. L. WALFORD: We have observed many granulomatous reactions in the hamster, and they did not occur in a pattern that correlated closely in all instances with the condition of the graft. Sometimes, one can observe these reactions early in first-set grafts and sometimes later.

I think this variability may be somewhat related to the hair follicle condition of the animal. One might say, however, that the hamster is able to form a good granulomatous reaction rather promptly, responding at least to the hair shaft material and to the sebaceous glandular material in the transplant. This is largely in the nature of a foreign body reaction, but it is a good granulomatous reaction. Whether such a type of foreign body granulomatous reaction would fulfill Chase's criterion for a delayed hypersensitivity reaction is perhaps unknown.

BILLINGHAM (*Wistar Institute*): It seems quite probable that these histological studies of chronic low-grade skin homograft reactions will yield evidence of two components of the host's response: one directed against the epidermis of the graft and the other against its dermis. In some of the grafts that Hildemann has described and illustrated, the entire graft epidermis may have been insidiously replaced long ago, and the inflammatory response observed may be directed entirely against the residue of the graft's dermis. It is even uncertain whether any living homologous mesenchymal cells are present. I have frequently observed that the smooth, hairless epidermis of skin homografts that are undergoing mild chronic reactions in hamsters is usually attached only very weakly to the underlying dermis. Presumably, this is usually native epithelium that is growing on an unstable bed.

ROGERS: I must give credit to my associate, Dr. Ballantyne, whose histological findings in these chronic reactions may help to amplify the preceding remarks. Ballantyne describes in a paper we now have in preparation what he calls, rather colorfully, the "Erlenmeyer-flask" shape of the dermal part of skin homografts undergoing the chronic "scar" type of rejection in closely related CFN rat parents and their offspring. The dermis of the graft remnant at this stage exhibits a peculiar shape similar to an Erlenmeyer flask, with a narrow upper dermal layer (or flask neck) and broad increasingly wide dermal layers as the layers get deeper and deeper, such that the broadest portion of the graft dermal remnant directly overlays the host bed. This strongly suggests to Ballantyne that most of the original epidermis and upper part of the dermal pad are reabsorbed and replaced by the host at a faster rate than the deeper portions of the dermal pad.

Reference

1. Saenz, A. 1956. Corps de Metchnikoff-Schaumann chez le hamster doré inoculé avec du BCG. Ann. inst. Pasteur. **90:** 751.

GENETICS OF SKIN TRANSPLANTATION AND AN ESTIMATE OF THE NUMBER OF HISTOCOMPATIBILITY GENES IN INBRED GUINEA PIGS

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Our present understanding of transplantation genetics is based principally on tumor transplantation in inbred strains of mice. From these studies a series of postulates emerged defining the genetic control of homograft compatibility (summarized by Snell, 1953). The fundamental principle is that graft survival obtains only if the recipient possesses all the genetically controlled transplantation antigens contained in the grafted tissue. Although the genetic, and hence antigenic, identity of donor and recipient satisfies this condition, such bilateral matching of antigens is not an essential requirement. A unilateral matching of graft antigens by the recipient is adequate to assure graft survival; host antigens, in addition to antigens present in the graft, appear not to compromise the survival of most normal tissue or tumor grafts. Transplants of immunologically competent cells, however, seem to require bilateral antigenic matching for continued survival of host or graft or both (Boyse, 1959).

The relatively few genetic studies of normal tissue grafts appear to justify the prevalent assumption that the genetic principles governing tumor grafts apply, with important qualifications, to normal tissue homografts as well. This inference is supported by studies utilizing spleen fragments (Little and Johnson, 1922; Bittner, 1936) and normal skin grafts (Billingham *et al.*, 1954; Prehn and Main, 1954; Eichwald *et al.*, 1957) in inbred strains of mice and their F_1 generation progeny. An estimate of the number of histocompatibility genes controlling splenic tissue transplants in mice was made by Bittner (1936), and a more recent estimate, based on an exhaustive study of parent strain skin grafts in F_2 generation mice, was reported by Barnes and Krohn (1957).

The elucidation of transplantation genetics in animals other than mice had to await the development of inbred strains. Taking advantage of two inbred strains of platyfish, Kallman and Gordon (1958) explored the genetics of fin transplantation. Loeb and Wright (1927), making early use of five families of guinea pigs inbred to nearly autogenous intrastrain compatibility, studied a variety of subcutaneously implanted organ fragments between these guinea pig families and their F_1 generation hybrids.

In view of the paucity of genetic studies involving normal tissue transplants, more particularly in animals other than mice, it was considered desirable to evaluate the genetics of guinea pig skin transplantation and to estimate the number of genetically independent transplantation antigens determining the fate of skin grafts between two inbred strains of guinea pigs.

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Materials and Methods

Two inbred strains of guinea pigs, Strains 2 and 13, were employed in this study. These strains were brother × sister mated over a 50-year period, and had been used earlier by Loeb and Wright (1927). In 1956 Jules Freund assembled animals of these two strains for the purpose of studying the relationship of genetic background to problems of hypersensitivity. Complete intrastrain histocompatibility was demonstrated (Bauer, 1958), and it was found that fur-bearing isografts within each of these 2 strains persisted for at least 20 weeks, whereas interstrain homografts were invariably rejected between 7 and 9 days after grafting.

F_1 generation hybrids were obtained by mating (approximately 20) Strain 13 females with Strain 2 males (1 male per group of 4 females) to obtain F_1^2 progeny; an equal number of animals was mated in the reciprocal relationship, Strain 2 females with Strain 13 males, to yield F_1^{13} progeny.

F_2 generation animals were obtained by mating F_1^2 males with F_1^2 females. Backcross animals (BC) were obtained by mating F_1^{13} males with Strain 13 females. It is interesting to note that none of these matings resulted in segregation of coat colors found in the multicolored parent strains.

All animals were maintained on a standard laboratory diet consisting of rabbit pellets, supplemented with raw cabbage three times weekly and water *ad libitum*. Young were weaned at the age of 4 weeks.

Animals weighed between 350 and 550 gm. at the time of grafting. Split-thickness skin grafts were used as test transplants and placed on full-skin-thickness graft beds prepared with a dermatome as described in detail previously (Bauer, 1958). Grafts were initially inspected 6 days after grafting (second-set grafts at 4 days) and daily thereafter until slough was completed; grafts persisting beyond 20 days were inspected at 2- to 3-day intervals. At each inspection color, thickening, and induration or any other changes of the grafts were recorded, and the onset of hair growth was noted.

In the present experiments sheets of split-thickness skin approximately 16 × 6 cm. were taken from each lateral thoracoabdominal aspect of donor animals, and each of these sheets was then cut into four 4 × 6 cm. grafts. Thus groups of 6 to 8 recipients could be tested with uniformly prepared grafts from a single donor.

The number of histocompatibility genes was estimated by determining the proportion of successful parent strain grafts in F_2 generation and backcross animals. Such an estimate is based on two major assumptions: (1) that histocompatibility genes segregate independently; and (2) that the antigenic product determined by each gene is singly sufficient to elicit graft rejection. As neither of these conditions necessarily obtains, only minimum numbers of unlinked histocompatibility gene loci can be detected, and loci carrying the same allele in the two strains escape detection. It can be shown that the proportion of successful grafts in F_2 animals is defined by $(3/4)^n$ (where n is the number of factors satisfying the assumptions mentioned above), and in backcross animals by $(1/2)^n$. It similarly follows that the proportion of F_2 animals accepting *both* parent strain grafts is also $(1/2)^n$.

Results

Isografts. Since the previous report (Bauer, 1958) skin isografts within each of the Strains 2 and 13 have been observed for more than 2 years without evidence of delayed adverse host reaction to any of the grafts, irrespective of the sex of donor or recipient.

Homografts. First-set interstrain homograft rejection times (compiled control data from several experiments) are given in TABLE 1. The mean graft survival times in the 2 reciprocal graft-recipient relationships are nearly identical at 7.7 ± 0.1 days. These data are based on the end point of histological necrosis of the epithelial layer of the skin graft. On gross inspection this end point coincides with peeling and detachment of the friable, thickened epidermis from the markedly indurated dermal elements. This reaction differs sharply from the rejection pattern of second-set skin homografts in two important respects: (1) second homografts applied between 7 and 200 days following the first grafting (from the same donor strain) show evidence of graft death much earlier (at 4 or 5 days after grafting), as shown in TABLE 2; (2) aside from this temporal difference, second-set grafts lack the thickening, induration, and color changes seen in first-set grafts and appear blanched white, remain thin, not vascularized, and without attachment to their granulation tissue beds.

TABLE 1
FIRST-SET HOMOGRAFT REJECTION

Donor strain	Recipient strain	No. of grafts applied	No. of grafts rejected on respective days				Mean graft survival (days)
			6	7	8	9	
13	2	62	1	20	34	7	7.7 ± 0.1*
2	13	42	1	14	26	1	7.6 ± 0.1

* Standard error of mean.

TABLE 2
SECOND-SET HOMOGRAFT REJECTION

Donor strain	Recipient strain	No. of grafts	No. of weeks between 1st and 2nd grafts	Reaction 4 or 5 days after 2nd grafting
13	2	3	1	"white graft"
13	2	6	2	"white graft"
13	2	3	3	"white graft"
13	2	10	10	"white graft"
13	2	4	16	"white graft"
13	2	7	28	"white graft"
Total		33		
2	13	9	2	"white graft"
2	13	6	10	"white graft"
2	13	5	26	"white graft"
Total		20		

Parent strain grafts to F_1 generation recipients. Twenty-two F_1 generation animals were grafted with 3 skin grafts each: one from each parent strain placed in adjacent positions in the same skin defect, and one graft from an F_1 generation donor on the opposite side of the animal. Sixty-three of these grafts were judged technically satisfactory and were followed for more than 80 days. The choice of donors and recipients was such as to represent various combinations of F_1^2 and F_1^{13} and parent strain animals as shown in TABLE 3. All grafts were fully accepted and fur-bearing for at least 80 days, and 36 grafts (12 F_1 recipients) were observed for more than 1 year without evidence of graft rejection.

By proper selection of sex and strain of graft donor and recipient, it was possible to evaluate potential transplantation antigens associated with either of the 2 sex chromosomes, x or y. Even in highly inbred animals with complete genetic homozygosity, the sex chromosomes of male animals are clearly heterozygous, and antigens associated with either sex chromosome are represented in males as haploids only, in contrast to the diploid representation of all antigens linked with the x chromosome in females. In the breeding of F_1 generation animals, further variations in sex chromosome combinations are possible because 2 sets of x and y chromosomes, one set from each parent strain, are involved. Thus all female F_1 hybrids resulting from crossing two inbred strains are identical in genetic composition, but male F_1 animals, although identical in autosomal composition, fall into 2 distinct groups on the basis of sex chromosome representation. F_1^2 males inherit the Strain 2 y-chromosome and the Strain 13 x-chromosome, whereas the alternate strain sex chromosomes are represented in male F_1^{13} animals; female F_1^2 and F_1^{13} animals inherit 1 x chromosome from each parent strain and are therefore identical in their sex chromosome as well as autosomal chromosome constitution. TABLE 4 is based on the same grafts tabulated in TABLE 3, but is arranged so as to illustrate the various x and y chromosome combinations. It will be noted that certain combinations test a potential incompatibility of graft antigens associated with y chromosomes of Strain 2 origin, whereas others test the potential antigenicity of x chromosomes of Strain 2 origin. Similarly, potential incompatibilities due to X or Y chromosomes of Strain 13 origin can be evaluated. In still other

TABLE 3

GRAFTS TO F_1 RECIPIENTS

Graft donor	Graft recipient	No. of grafts applied and surviving >80 days
Strain 2	F_1^{2}*	18
	F_1^{13}†	4
Strain 13	F_1^2	17
	F_1^{13}	4
F_1^2	F_1^2	12
	F_1^{13}	4
F_1^{13}	F_1^2	4

All grafts were fully accepted and fur-bearing. Some of the grafts were observed for more than 1 year.

* F_1^2 denotes male Strain 2 × female Strain-13 hybrid.

† F_1^{13} denotes male Strain 13 × female Strain-2 hybrid.

combinations the graft carries the x chromosome of Strain 2 origin and the Y chromosome of Strain 13 origin, whereas the recipient possesses X and y chromosomes each from the opposite strain (Xy) as that represented in the graft (xY). In the latter combination there is no *strain-specific* representation in the recipient of either the x or y chromosome carried in the graft and thus potential incompatibility due to both sex chromosomes could arise. In fact, however, no such incompatibilities were noted during an observation period of more than 80 days in any of the combinations tested. These results suggest that neither the x nor the y chromosome is associated with transplantation antigens in the guinea pig strains tested.

F_1 to parent strain grafts. The rejection times of F_1 hybrid grafts containing a haploid number of genes foreign to *either* parent strain were evaluated by grafting 41 Strain 2 (Group A, TABLE 5) and 17 Strain 13 (Group C) recipients.

TABLE 4
STRAIN-SPECIFIC DIFFERENCES IN SEX CHROMOSOMES

Sex chromosomes of graft recipients	Sex chromosomes of graft donors			
	Strain 2 male xy	Strain 13 male XY	F_1^2 male Xy	F_1^{13} male xY
F_1^2 males Xy	x (12)*	Y (12)	0 (4)	xY (4)
F_1^{13} males xY	y (4)	X (4)	Xy (4)	0 (0)
Females F_1^2 or F_1^{13} xX	y (6)	Y (5)	y (4)	Y (0)

Small letters (x and y) represent Strain-2 sex chromosomes.
Capital letters (X and Y) represent Strain-13 sex chromosomes.
Figures in parentheses give the number of grafts observed in each particular graft donor combination.
* Letters indicate the sex chromosome in the graft which is not present in the recipient and therefore a potential source of incompatibility.

It may be noted that the F_1 graft rejection times are scattered over a much wider range (6 to 14 days) than the interstrain homografts (TABLE 1) containing a diploid number of incompatible genes. Of 104 interstrain homografts, none survived beyond the ninth postgrafting day, whereas 21 per cent of F_1 grafts survived beyond 9 days (12 of 58 grafts; Groups A and C, TABLE 5).

To examine the possibility that F_1 grafts constitute an inadequate (submaximal) antigenic stimulus to parent strain recipients, 22 parent strain guinea pigs (Groups B and D) were grafted simultaneously with 2 grafts—1 F_1 graft and 1 parent strain homograft. This double grafting was intended to potentiate the dosage of incompatible graft antigens. Although F_1 graft survival times appear to have been shortened in Group B, 14 per cent of F_1 grafts (3 of 22; Groups B and D) still survived beyond 9 days, despite the fact that all simultaneously applied interstrain homografts were rejected at the usual time of less than 9 days (not shown in the table). Considering the 22 "doubly grafted" animals (Groups B and D) on an individual basis, 13 of the 22 recipients rejected the F_1 graft between 1 and 6 days later than the simultaneously applied interstrain grafts, and the remaining 9 animals rejected both grafts on the same day; in no instance was an F_1 graft the first of the 2 grafts to be rejected by any one animal.

Comparing the 55 $F_1 \rightarrow$ Strain 2 grafts (Groups A and B combined) with the 62 Strain 13 → Strain 2 grafts (TABLE 1) in terms of the number of grafts surviving beyond the ninth postgrafting day (11 of 55 versus 0 of 62), a statistically significant difference between the 2 graft samples is noted ($chi^2 = 11.21$; $P < 0.001$). Comparing the $F_1 \rightarrow$ Strain 13 grafts (Groups C and D combined) with the Strain 2 → Strain 13 grafts (TABLE 1) on the same basis, again a statistically significant difference is found ($chi^2 = 4.58$; $P < 0.05$).

TABLE 5
REJECTION TIMES OF F_1 GRAFTS BY PARENT-STRAIN RECIPIENTS

Group	Recipient strain	Graft donor strain	No. of F_1 grafts rejected on respective postgrafting days									No. of F_1 grafts
			6	7	8	9	10	11	12	13	14	
A	2	$F_1{}^2$		2	7	2	2			1	2	16
		$F_1{}^{13}$	2	11	6	1	1	3		1		25
Total			(2)	(13)	(13)	(3)	(3)	(3)		(2)	(2)	(41)
B	2	$F_1{}^2$ and 13		5	4			1				10
		$F_1{}^{13}$ and 13		2	1	1						4
Total*				(7)	(5)	(1)		(1)				(14)
C	13	$F_1{}^2$		2	1		1		1			5
		$F_1{}^{13}$	1	7	4							12
Total			(1)	(9)	(5)		(1)		(1)			(17)
D	13	$F_1{}^2$ and 2			1	2	1					4
		$F_1{}^{13}$ and 2			3						1	4
Total*					(4)	(2)	(1)				(1)	(8)

* Total F_1 grafts; Strain-2 and Strain-13 grafts not tabulated.

Another attempt to elucidate the role of graft antigen dosage in the prolonged survival of F_1 to parent strain grafts is presented in TABLE 6. The relationship of graft antigen dosage to graft survival time was examined by varying the size of grafts within the range of 6 cm.^{2} and 68 cm.^{2} of graft surface area per recipient guinea pig. No appreciable difference in graft survival times is detectable within a tenfold range of graft sizes. As the size of all $F_1 \rightarrow$ parent strain grafts (of TABLE 5) was 15 cm.^{2} (3 × 5 cm.), the prolonged survival of F_1 grafts simply on the basis of inadequate antigen dosage would appear unlikely.

Parent to F_2 grafts. Each of 27 F_2 generation guinea pigs received 2 technically satisfactory skin homografts, 1 from each of the parent strains (2 and 13). The 2 grafts were placed in adjacent positions of the same graft bed, on the right thoracoabdominal aspect of each F_2 recipient. Twenty-one Strain 2 grafts (78 per cent) were rejected between 8 and 52 days after grafting, and 19 Strain 13 grafts (70 per cent) were rejected between 7 and 46 days. The distribution of graft rejection times is given in TABLE 7.

Grafts rejected in the first 15 to 20 days exhibited a reaction very similar to

TABLE 6

INTERSTRAIN HOMOGRAFT DOSAGE

Graft recipient strain	Graft donor strain	Size of graft cm.^{2}	No. of grafts rejected on respective postgrafting days		
			7	8	9
2	13	56–68		1	1
2	13	24	1	1	
2	13	12	1		1
2	13	6	1	1	
Totals			3	3	2
13	2	58	1	1	
13	2	24	2		
13	2	12	1	1	
13	2	6	1	1	
Totals			5	3	

TABLE 7

REJECTION TIMES OF PARENT-STRAIN GRAFTS BY F_2 HYBRIDS

Graft donor	No. of grafts rejected on respective postgrafting days																									
	4	6	8	10	12	14	16	18	20	22	24	26	28	30	32	34	36	38	40	42	44	46	48	50	52	54
First-set grafts																										
Strain 2			2	3	1	3			1	1	1	1				1	2	1				2	1		1	
Strain 13			4	1		2	2	1	2			2	1	1						1	1	1				
Second-set grafts																										
Strain 2	4*	1	1			1				1		1	1	1												
Strain 13	5*	1			1	1		1		1																

Graft donor	Mean survival time (days)	Total grafts	Per cent graft survival			
			Observed	Calculated for differences of (genes)		
				4	5	6
First-set grafts						
Strain 2	25	21	22%		24%	18%
Strain 13	21	19	30%	32%	24%	
Second-set grafts						
Strain 2	12	11				
Strain 13	11	10				

* "White" grafts.

interstrain homografts (blue-red discoloration, marked thickening and induration, fragmentation and peeling of the necrotic epithelium). The behavior of grafts rejected after the twentieth postgrafting day was rarely found to undergo the rapid and distinct changes seen in the first few weeks. In such grafts, changes accompanying breakdown often consisted of no more than a moderate induration and a dry flaky desquamation. This gradually arising, eczematous reaction usually lasted for a period of 5 to 10 days and, on subsiding, there remained a very smooth, white, firm scar tissue extending over an area somewhat smaller than the original graft. Further cicatricial contraction was usually slight, and hair growth did not occur.

Such slowly rejected grafts are clearly distinct from fully accepted, pliable, furbearing grafts, but it was often difficult to pinpoint the exact day on which the graft could be adjudged completely rejected. Although the initiation of hair growth was found to be an unreliable indication of the future course of the graft, absence of hair growth at the end of 3 weeks was often the earliest sign of latent incompatibility (regeneration of hair growth in split-thickness isografts normally begins between fourteen and twenty days).

From a total of 27 Strain-2 grafts, 6 (22 per cent) survived for at least 80 days (5 of these were observed for 120 days, and 4 were observed for 180 days—TABLE 8). Of 27 Strain-13 grafts, 8 (30 per cent) survived for at least 80 days (6 of these grafts were observed for 120 days, and 4 for 180 days). Three of the 27 F_2 recipients (11 per cent) tolerated parental grafts from *both* parent strains for at least 120 days (2 of these were observed for more than 180 days).

If the value n in $(3/4)^n$ be calculated so that $(3/4)^n$ = the observed proportion of successful grafts, it is found that at least 5 and possibly 6 independently segregating transplantation antigens control the incompatibility of Strain-2 grafts (if $n = 5$, takes = 24 per cent; if $n = 6$, takes = 18 per cent); similarly, at least 4 genetically controlled antigens are responsible for Strain 13 graft rejection (if $n = 4$, takes = 32 per cent). *See* TABLE 7.

Calculating n in $(1/2)^n$ so as to approximate the observed proportion of F_2 animals accepting both parent strain grafts (3 in 27 = 11 per cent), at least 4 independently segregating antigen differences are required to account for incompatibilities between Strains 2 and 13 (if $n = 3$, takes = 13 per cent; if $n = 4$, takes = 6 per cent).

Second-set parent to F_2 grafts. Sixteen of the 27 F_2 guinea pigs were grafted with a Strain-2 and Strain-13 graft for a second time, on the left thoracoabdominal aspect. There was an interval of 50 to 60 days between the first and second grafting. The distribution of second-set rejection times is also shown in TABLE 7. The mean second-set graft survival times of each donor strain are less than one half the corresponding first-set mean survival times.

TABLE 8 gives a comparative listing for each individual F_2 recipient of the 2 sets of grafts (first and second set) from each donor strain. It will be noted that all F_2 animals that rejected the first-set grafts also rejected the corresponding second-set skin grafts, but only 4 of 11 Strain-2 grafts (36 per cent) and 5 of 10 Strain-13 grafts (50 per cent) were found to be "white grafts" of the type consistently seen in second interstrain homografts (TABLE 2). Although the second-set survival times in 2 F_2 recipients exceeded those of the corresponding first-set grafts, a general proportionality between first- and second-set survival times in most individual recipients is evident (TABLE 8). All animals bearing surviving first grafts also accepted corresponding second grafts. In no instance were surviving first-set grafts compromised by the application of a second graft from the same donor strain.

TABLE 8

COMPARISON OF FIRST- AND SECOND-SET PARENT-STRAIN GRAFTS IN INDIVIDUAL F_2 RECIPIENTS

Strain 2 graft rejection (days)		Strain 13 graft rejection (days)	
First set	Second set	First set	Second set
8	<4	18	<4
8	<4	30	13
9	<4	9	6
9	*	13	*
10	*	Survival (>80)	*
11	*	7	*
13	6	7	<4
13	*	27	*
14	13	15	*18*
19	8	42	22
22	<4	19	<4
24	*26*	Survival (>180)	Survival (>120)
26	*	25	*
33	*	46	*
35	*	Survival (>80)	*
36	22	Survival (>180)	Survival (>120)
38	*	44	*
45	27	15	11
46	*	7	*
47	*	26	*
52	29	Survival (>120)	Survival (>60)
Survival (>180)	Survival (>120)	13	<4
Survival (>180)	Survival (>120)	8	<4
Survival (>80)	*	20	*
Survival (>120)	Survival (>60)	Survival (>120)	Survival (>60)
Survival (>180)	Survival (>120)	Survival (>180)	Survival (>120)
Survival (>180)	Survival (>120)	Survival (>180)	Survival (>120)
Number and per cent of grafts surviving beyond 80 days			
6 (22%)		8 (30%)	

* Not done.
Each horizontal line represents data of a single F_2 recipient.
Key: <4 days implies "white" graft rejection.
Italicized figures denote second-set graft survival that exceeds survival time of first-set graft.

Parent-strain grafts to backcross recipients. Seventeen (F_1^{13} × Strain 13) backcross (BC) guinea pigs were grafted with 1 skin homograft from each parent strain. All but 1 Strain-2 graft were rejected between 8 and 39 days after grafting. The distributions of graft rejection times are given in TABLE 9. The mean survival time of Strain-2 grafts → BC recipients is considerably shorter than that of Strain-2 → F_2 grafts (14 days versus 25 days).

One of the 17 BC guinea pigs (6 per cent) retained its Strain-2 graft for more than 100 days. A second Strain-2 graft applied 50 days later has also survived for at least 50 days. Calculating the n value in $(\frac{1}{2})^n$ to correspond to the proportion of observed graft survival (1 in 17 = 6 per cent), the result indicates that no less than 4 independently assorting antigens participate in Strain-2 graft rejection (if $n = 4$, takes = 6 per cent).

All 17 Strain-13 BC grafts have survived for at least 100 days. As Strain 13 represents the parent strain to which the F_1 progeny was "crossed back," these BC animals possess at least 1 complete haploid set of Strain-13 histocompatibility genes and would therefore be expected to accept Strain-13 skin grafts permanently.

Strain-2 second-set grafts were applied to all 17 BC recipients. Sixteen of the grafts were rejected between 4 and 18 days (TABLE 9) and 12 (75 per cent) resulted in white graft reactions. Each BC recipient rejected its second Strain-2 graft more rapidly than the first graft.

TABLE 9
PARENT-STRAIN GRAFTS TO (F_1 × 13) BACK-CROSS RECIPIENTS

Graft donor	No. grafts rejected on respective postgrafting days									Total grafts	Grafts surviving beyond 100 days		Mean survival time (days)
	4	6 8	10	12	14	16	18	20	39		Number	Per cent	
First-set grafts													
Strain 2		2	2	2	4	2	1	2	1	17	1	6%	14
Strain 13										17	17	100%	>100
											Grafts surviving beyond 50 days		
Second-set grafts													
Strain 2	12*	1	1		1		1			17	1	6%	<4

* "White" grafts.

Discussion

Skin grafts present a number of advantages over tumor or other normal tissue grafts in the study of transplantation genetics. In general, these advantages concern the relative ease of orthotopic transplantation and the great convenience in continual observation of graft survival and function. However, the particular merit of skin grafts resides in their effectiveness to induce lasting donor-specific transplantation immunity and in their exquisite sensitiveness to detect such an immune response.

The choice of tumor grafts for histocompatibility evaluations is a less satisfactory one. The proclivity of tumor grafts to override host reactions that are submaximal or slow in arising, but to which normal tissue grafts would eventually succumb, constitutes the major objection to their use as test grafts. Furthermore, the successful "take" of a tumor graft (manifested by killing its host) gives no assurance that, in fact, graft-host compatibility did prevail. For these reasons tumor-test grafts must be judged as less sensitive and less reliable indicators of tissue compatibility. It follows that, if tumor grafts be used as test transplants, a numerical underestimation of transplantation antigens probably would result. This supposition is indeed upheld by experimental findings; the most specific tumors are found to be controlled by nearly as many antigens (Little and Tyzzer, 1916, estimated 14 or 15 controlling genes) as normal skin grafts (Barnes and Krohn, 1957, estimated 14 to 17 controlling genes), but the least specific tumors seem to require no more than 2 or 3 controlling histocompatibility genes (Snell, 1953).

Other complications attend the use of immunologically competent cells as test grafts. That lymphoid tissue grafts appear to be adversely affected by incompatible antigens in either donor or host and would most likely yield an overestimate of the number of controlling transplantation antigens is suggested by the work of Boyse (1959). Investigating the fate of antibody-producing mouse spleen cells transplanted intraperitoneally into F_1 hybrid animals, Boyse found that parental spleen-cell grafts survived for only brief periods (2 to 5 weeks) on F_1 hosts that tolerate skin grafts from the same parent strain donors permanently. As the parent strain spleen grafts introduced no antigens foreign to the F_1 hosts, Boyse suggests that graft failure must result from the overwhelming antigenic stimulus that the foreign F_1 host antigens present to the graft.

The results of the present study, suggesting permanent survival of parent strain guinea pig skin grafts in F_1 recipients, are in agreement with similar reports involving mice, guinea pigs, and fish. It is further apparent that F_1 animals (F_1^2 and F_1^{13}) resulting from the 2 possible reciprocal parent strain crosses, are equally receptive to grafts from both parent strains (TABLE 3), as well as to grafts from either F_1^2 or F_1^{13} donors. It is interesting to note that grafts from the 2 parent strains placed in adjacent positions within the same graft bed of an F_1 recipient healed firmly at their common line of contact. This observation clearly illustrates the lack of mutual incompatibility of 3 coexisting antigenically dissimilar tissues (skin of F_1, Strain 2, and Strain 13—each devoid of the capacity for immune reaction) and re-emphasizes the immunological basis of tissue incompatibility.

The work of Eichwald *et al.* (1957), suggesting a sex-linked transplantation antigen, prompted the evaluation of this possibility in guinea pigs. These authors reported delayed rejection of tail skin grafts from male mice to females of the same inbred strain, as well as the rejection of male parent strain grafts by female F_1 recipients. More recently it was shown (Eichwald *et al.* 1958; Zaalberg, 1959) that in mice this sex-linked transplantation antigen is most likely Y chromosome-linked, is not strain-specific, and is found in all male tissues tested and in males of all mouse strains tested. Further, it was shown that females of all mouse strains do not respond to this antigen and that this "male factor" is a relatively weak transplantation antigen (skin graft rejections between 12 and 200 days were reported). Attempts to show *strain-specific* antigenic differences associated with the X chromosome in mice have not been successful.

In the present study it was attempted to determine whether a Y chromosome-linked antigen could be detected in guinea pigs and, if so, whether the Y-linked antigen is merely sex-specific (that is, antigenic to females only) or whether additional strain specificity, determined by the strain of origin, prevails. The grafting combinations shown in TABLE 4 are designed to test the strain-specific antigenicity of x *and* y chromosomes. Male grafts to female F_1^2 or F_1^{13} recipients evaluate Y-chromosome antigenicity per se, whereas male grafts to male F_1^2 and F_1^{13} recipients evaluate Y-chromosome antigenicity attributable to strain-specific differences. Thus if such *strain-specific* antigen differences were to exist, F_1^{13} males would reject grafts from Strain-2 males (strain-specific y incompatibility), and would reject grafts from Strain-13 males on the basis of strain-specific x incompatibility.

No such incompatibilities were noted, however. Grafts in all combinations tested were fully accepted, and no male to female graft incompatibility was observed during an observation period of 80 days.

Apparently conflicting evidence surrounds the question whether F_1 grafts, containing only a single (haploid) dose of genes foreign to parent strain recipients, are less antigenic and survive longer than grafts containing a double (diploid) dose of foreign genes. Billingham *et al.* (1954) were unable to demonstrate in mice a difference between survival times of skin grafts containing a haploid dose and grafts containing a diploid dose of incompatible genes. However, Prehn and Main (1954), also working with mice, reported a significantly prolonged F_1 graft survival (>200 days) in one of three $F_1 \rightarrow$ parent strain grafting combinations. It may be significant that prolonged F_1 graft survival was observed in a combination carrying the same allele at the strong H-2 locus.

Kallman and Gordon (1958) also observed markedly prolonged survival of $F_1 \rightarrow$ parent-strain fin grafts in fish. These authors further observed that if interstrain homografts were transplanted to fish that had not yet rejected the F_1 grafts, no appreciable acceleration of the F_1 graft rejection could be detected despite the rapid rejection of every interstrain homograft. On the basis of the survival of F_1 grafts (haploid dose of incompatible genes) extending beyond the rejection time of simultaneously applied interstrain homografts (diploid dose of incompatible genes), Kallman and Gordon suggest that the presence in F_1 grafts of 1 (haploid) set of chromosomes compatible with the parental host "protects" F_1 grafts against rapid destruction by host antibodies.

The findings of the present study involving guinea pigs are congruous with the observations made by Kallman and Gordon in fish. Some of both types of F_1 skin grafts (F_1^2 and F_1^{13}) were found to survive for significantly longer periods in both parent strain recipients (TABLE 5). Operationally, the prolonged survival of F_1 grafts was considered from 2 points of view: (1) an insufficient antigenic stimulus by a haploid dose of incompatible antigens may result in a submaximal immune response by the host, or (2) cells containing 1 set (a haploid number) of incompatible and 1 set of host-specific (compatible) chromosomes may be less vulnerable to an immune response by the host. The data present no evidence in support of the former possibility; suggestive evidence against the first assumption is found in (1) the prolonged survival of some F_1 grafts in hosts grafted simultaneously with an interstrain homograft (known to elicit an adequate immune response), and (2) the lack of an appreciable graft dosage effect (TABLE 6) over a range that would be expected to include the dosage differential of F_1 and interstrain grafts,* and (3) the accelerated second-set rejection pattern (white graft response) of interstrain homografts applied after F_1 graft rejection (unpublished data).

Observations in support of the second assumption consist of: (1) the prolonged survival of some F_1 grafts in hosts rejecting simultaneously applied interstrain homografts, (2) the finding that, whenever there is a difference in rejection times between the F_1 graft and the interstrain homograft carried by the same host, the interstrain graft is the first to be rejected.

The collective evidence would seem to justify the conclusion that skin grafts possessing a chromosomal and hence antigenic constitution of which one half is foreign and the other half is host-specific can in some cases withstand for short periods a degree of host immunity to which homozygous interstrain grafts succumb. Whether the prolonged survival of such heterozygous grafts results in a quantitative increase in the immune response until a degree adequate for F_1 graft rejection is attained requires further study.

Only 3 estimates of the number of independently segregating transplantation antigens based on normal tissue grafts were reported: 2 studies involve mice and 1 is based on fish. Investigations by Bittner (1936) indicating that 10 or 11 factors control transplants of spleen fragments in mice suffer from the technical complications associated with the use of lymphoid tissue. A fully competent study of this type, reported by Barnes and Krohn (1957), indicates that 2 of 120 grafts from 1 parent strain and 1 of 154 grafts of the other parent strain survived for more than 100 days in F_2 recipient mice. Calculations based on these 3 surviving grafts suggest that no less than 14 independently segregating genes control the incompatibility of skin grafts from 1 parent strain and that no less than 17 genes are responsible for rejection of grafts from the other strain. A third study, reported elsewhere in this monograph by Kallman, indicates that at least 3 histocompatibility genes are involved in the rejection of fin transplants between 2 closely related inbred strains of fish.

Numerical estimation of transplantation antigens in guinea pigs, based on the present study, fall into a somewhat lower range than those reported in similar studies involving mice; the transplantation of Strain-2 skin grafts is controlled by at least 5 (possibly 6) independently segregating, genetically determined antigens (based on 6 grafts surviving more than 80 days), and Strain-13 skin grafts are associated with at least 4 genetically controlled antigens (based on 8 grafts surviving more than 80 days). It may well be erroneous to conclude from these findings that fewer antigens are concerned with the transplantation of tissues in guinea pigs than in mice. There is reason to believe that the 2 guinea pig strains involved in this study may well be genetically more similar to each other than to other commonly available random bred colonies. This inference would appear reasonable in view of the fact that both strains were selected originally from the same genic pool of a single random-bred colony of guinea pigs (Wright, 1922). Genetic similarity between the 2 inbred strains is suggested further by the finding (unpublished) that the 2 strains *share* at least 1 strong transplantation antigen not represented in Hartley guinea pigs (random bred, albino).

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* It may be well questioned whether it is justifiable to compare gene dosage effects of homozygous and heterozygous (F_1) cells in terms of graft size (that is, antigen dosage of F_1 graft with area 15 cm.^{2} = (?) antigen dose of interstrain graft with area 7.5 cm.^{2}).

On examination of the distribution of graft rejection times in F_2 recipients (TABLE 7), a periodic clustering of graft sloughs becomes apparent. Although this apparent periodicity may represent a mere chance occurrence related to the small number of animals, it is tempting to suggest a possible relationship between the various clusters of graft rejection times and the individual transplantation antigens. Such a relationship is, at least, conceivable on purely theoretical grounds; if it be postulated that each histocompatibility gene determines antigens differing in ability to induce graft rejection (that is, the latency time required for graft rejection), the independent assortment of such antigens in F_2 generation animals could be expected to result in bursts of graft rejection temporally related to the latency period of each antigen or combinations of antigens. In support of this series of inferences based on scant evidence, it may be noted that genes determining antigens that require varying periods of time to induce graft rejection are well documented in mice (Snell, 1957).

This investigation endeavors to elucidate in guinea pigs some of the transplantation principles studied extensively in mice. A better understanding of these principles in a species that, next to man, is most susceptible to cell-mediated immune states lends itself to numerous applications. A re-examination of the passive transfer of cell-mediated hypersensitivity reactions (discussed perspicuously by Chase, 1959) under conditions that eliminate the interference of homograft immunity against the transferred cells would indeed seem to hold much promise.

Summary

First-set interstrain split-thickness skin homografts between 2 strains of inbred guinea pigs are rejected between 6 and 9 days after grafting; second-set homografts, applied between 7 and 180 days after first grafting, remain unvascularized and result in nonviable "white grafts"; these two rejection patterns are sharply distinct.

F_1 hybrids accept skin grafts from both parent-strains as well as from other F_1 guinea pigs permanently; skin grafts from the 2 mutually incompatible parent strains applied in adjacent locations onto F_1 hosts heal completely at their common line of junction.

Neither of the sex chromosomes, x or y, appears to be associated with transplantation antigens.

F_1 skin grafts are rejected by parent-strain recipients between 6 and 14 days after grafting; about 20 per cent of F_1 grafts survive beyond 9 days. Possible mechanisms of prolonged F_1 survival are evaluated.

Homograft survival time is not affected by varying the graft size between 6 and 68 cm.^{2}

Seventy-eight per cent of Strain-2 grafts and 70 per cent of Strain-13 grafts are rejected by F_2 recipients between 8 and 52 days after grafting.

Histocompatibility gene estimates, based on the proportion of parent-strain grafts surviving in F_2 and back-cross animals and on the proportion of F_2 animals retaining both parent-strain grafts indicate that no less than 6 genes control the rejection of Strain-2 grafts and no less than 4 genes control the rejection of Strain-13 grafts.

References

Barnes, A. D. & P. L. Krohn. 1957. The estimation of the number of histocompatibility genes controlling the successful transplantation of normal skin in mice. Proc. Roy. Soc. London. **B146:** 505–526.

Bauer, J. A., Jr. 1958. Histocompatibility in inbred strains of guinea pigs. Ann. N. Y. Acad. Sci. **73**(3): 663–672.

Billingham, R. E., L. Brent & P. B. Medawar. 1954. Quantitative studies on tissue transplantation immunity. I. The survival times of skin homografts exchanged between members of different inbred strains of mice. Proc. Roy. Soc. London. **B143:** 43–58.

Bittner, J. J. 1936. The transplantation of splenic tissue in mice. Public Health Repts. U. S. **51:** 244.

Boyse, E. A. 1959. The fate of mouse spleen cells transplanted into homologous and F_1 hybrid hosts. Immunology. **2**(2): 170–181.

Chase, M. W. 1959. Models of hypersensitivity studies. *In* Cellular and Humoral Aspects of the Hypersensitive States. : 251–278. H. S. Lawrence, Ed. Hoeber-Harper. New York, N. Y.

Eichwald, E. J., C. R. Silmser & N. Wheeler. 1957. The genetics of skin grafting. Ann. N. Y. Acad. Sci. **64**(5): 737–740.

Eichwald, E. J., C. R. Silmser & I. Weissman. 1958. Sex-linked rejection of normal and neoplastic tissue. I. Distribution and specificity. J. Natl. Cancer Inst. **20:** 563–575.

Kallman, K. D. & M. Gordon. 1958. Genetics of fin transplantation in xiphophorin fishes. Ann. N. Y. Acad. Sci. **73**(3): 599–610.

Little, C. C. & B. W. Johnson. 1922. The inheritance of susceptibility to implants of splenic tissue in mice. Proc. Soc. Exptl. Biol. Med. **19:** 163.

Little, C. C. & E. E. Tyzzer. 1916. Further experimental studies on the inheritance of susceptibility to a transplantable tumor, carcinoma (J.W.A.) of the Japanese waltzing mouse. J. Med. Research. **33:** 393–453.

Loeb, L. & S. Wright. 1927. Transplantation and individuality differentials in inbred families of guinea pigs. Am. J. Pathol. **3:** 251.

Prehn, R. T. & J. M. Main. 1954. A comparison between heterozygous and homozygous skin homografts. J. Natl. Cancer Inst. **15:** 191–194.

Snell, G. D. 1953. The genetics of transplantation. J. Natl. Cancer Inst. **14:** 691–700.

Snell, G. D. 1957. The homograft reaction. Ann. Rev. Microbiol. **11:** 439–458.

Wright, S. 1922. The effect of inbreeding and cross-breeding on guinea pigs. U. S. Dept. Agr. Bull. **No. 1090.**

Zaalberg, O. B. 1959. An analysis of the Eichwald-Silmser effect. Transplantation Bull. **2:** 433–435.

AGE FACTOR AND INDUCTION OF IMMUNOLOGICAL TOLERANCE TO MALE SKIN ISOGRAFTS IN FEMALE MICE SUBSEQUENT TO THE NEONATAL PERIOD*

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Sex-linked histoincompatibility to male skin isografts in females among some inbred strains of mice was previously reported by Eichwald and Silmser,[1] Prehn and Main,[2] and Short and Sobey,[3] and confirmed by many others.[4-8]

Eichwald *et al.*,[9] Hauschka,[10] and Snell[11] have interpreted the rejection of the male skin by the female of the same inbred strain as an immune response caused by the recipient's lack of the donor's isoantigen, a situation determined by a gene located on the Y chromosome.

Eichwald *et al.*,[12] as well as Sachs and Heller[6] have proposed that this phenomenon is present to a degree in most inbred strains.

Due to these reports, experiments were conducted in our laboratory to determine if mice of other inbred strains raised in our colony exhibited the sex-linked histoincompatibility phenomenon.

TABLE 1 illustrates the results obtained using Z (C3H), BALB/c, Ce, and NH inbred strains of mice. The results demonstrate that only the Z (C3H) and BALB/c do not show the Eichwald-Silmser phenomenon of sex histoincompatibility. The male skin isograft was always accepted by the females of the Z (C3H) strain, while there was an 85 per cent acceptance of male skin by the BALB/c females.

In contrast, the females of the Ce and NH inbred strains definitely exhibit the sex histoincompatibility.

As expected, a uniform acceptance in all strains was observed when skin transplants were attempted between female → male, male → male, and female → female.

Since old female recipients did not often accept the skin isografts from old male donors, TABLE 2 offers supportive evidence that sex histoincompatibility does exist in the Ce and NH strains.

The findings in the Z (C3H) and BALB/c are in agreement with Prehn and Main,[2] but not with the observations of Sachs and Heller,[6] who found sex histoincompatibility in both of these strains. The discrepancies may arise from genetic differences of our Z (C3H) and BALB/c animals in comparison with the strains used by Sachs and Heller. The mice were maintained for many generations according to strict inbreeding procedures by our colleague John J. Bitt-

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ner. In 1956 they were separated from Bittner's colony and since then have been maintained in our laboratory by rigorous inbreeding.

Further experimentation has demonstrated the immunological nature of the Eichwald-Silmser phenomenon. Acquired tolerance to male skin isograft was induced in females of the A and C57BL (subline 1) strains by injecting them at birth with male viable spleen cells as carried out by Mariani *et al.*[5] Identical results were obtained in the C57BL strain by Billingham *et al.*[4]

TABLE 1

SKIN ISOGRAFTS FROM MALE TO FEMALE, FEMALE TO MALE, MALE TO MALE, AND FEMALE TO FEMALE IN SEVERAL INBRED STRAINS OF MICE*

Strain	No. takes/ total number grafted	Per cent	Recipient age range (days)	Donor age range (days)	No. takes/ total number grafted	Per cent	Recipient age range (days)	Donor age range (days)
	♂ to ♀				♀ to ♂			
Z(C3H)	21/21	100	33–62	33–51	16/16	100	33–42	33–34
Ce	6/13	46	38–62	45–53	7/7	100	40–51	40–51
BALB/c	17/20	85	54–78	54	20/20	100	34–70	43–70
NH	6/20	30	36–58	32–63	8/9	89	36–58	32–63
	♂ to ♂				♀ to ♀			
Z(C3H)	23/25	92	33–62	33–62	19/19	100	33–38	33–38
Ce	16/16	100	35–55	35–55	7/7	100	37–52	37–52
BALB/c	14/14	100	34–65	34–66	23/23	100	36–66	36–66
NH	21/21	100	35–59	35–59	11/12	92	32–57	32–57

* Successful grafts were maintained during the entire period of observation, ranging from 5 months to 1 year following grafting.

TABLE 2

SKIN ISOGRAFTS FROM OLD MALES TO OLD FEMALES OF SEVERAL INBRED STRAINS*

Strain	No. takes/total no. grafted	Recipient		Donor	
		Age range (days)	Average age (days)	Age range (days)	Average age (days)
Z(C3H)	9/9	151–332	185	274–365	335
Ce	2/6	365	365	365	365
BALB/c	12/14	158–310	196	169	169
NH	3/17	365	365	173–365	254

* Successful grafts were maintained during the entire period of observation, ranging from 5 months to 1 year following grafting.

However, findings in our laboratory have indicated that in female animals of the A strain not injected at birth with male spleen cells, the age of the recipient and donor at the time of skin transplant might be an additional factor to be considered in the phenomenon of male skin isograft rejection.[13,14]

In studying the age factor, 5 groups of inbred mice from the A strain and 2 groups from the C57BL (subline 1) strain were set up with male donors and female recipients. The female was grafted on its dorsal surface with a 2 × 2 cm. full-thickness abdominal skin graft, dissected free of subcutaneous fat, and taken from the male donor as described by Martinez *et al.*[15] To facilitate determination of subsequent success or failure of the graft, it was turned 180°, since on a successful transplant the hair growth is in a direction opposite to hair growth of the host. All grafted animals were placed in individual plastic boxes and were observed from 5 to 12 months.

The skin isograft acceptance from male to female in the A strain is shown in TABLE 3. Of special interest is the comparison of the isotransplant acceptance in Groups I, II and III where the donors and recipients are of the same age range: 1 to 1½ months, 2 to 2½ months, and 7 to 9 months, respectively. The apparent wide differences in percentage of acceptance, 75 per cent in Group I, 40 per cent in Group II and 0 per cent in Group III are statistically significant. The difference between Groups I and II is statistically significant at the 5 per cent level, and at the 1 per cent level between Groups I and III and between Groups II and III.

TABLE 3

SKIN ISOGRAFTS FROM MALE TO FEMALE IN STRAIN A MICE OF DIFFERENT AGES

Groups	Male donor age (days)*	Female recipient age (days)*	Total no. isografts	Successful grafts	
				Number	Per cent
I	42 ± 1	36 ± 1	20	15	75
II	46 ± 2	59 ± 1	20	8	40
III	216 ± 23	274 ± 16	17	0	0
IV	43 ± 2	228 ± 15	15	7	47
V	280 ± 20	37 ± 1	20	13	65

* Mean and standard deviation.

Some interesting correlations can be made with Groups IV and V when the age of either the donor or the recipient is altered. Perhaps the growth potential of the donor skin transplanted is evident when one compares the 47 per cent successful acceptance of Group IV, when the old recipients are receiving transplants from young donors, to 0 per cent in Group III where old recipients receive transplants from old donors. However, the difference in percentage of graft acceptance between Group V (young recipients, old donors) and Group I (young recipients, young donors) is not significant.

These results indicate the importance of age as a factor of successful skin isotransplants in both male donors and female recipients of the A strain, although it appears more clear-cut when the age of the recipients is varied.

TABLE 4 gives the male to female isograft results for the C57BL (subline 1) strain. In the young (29- to 34-day-old group) there was a successful graft incidence of 19 per cent, closely approximating the 13 per cent incidence for the 180-day-old group. Although the ages of both recipient and donor do not influence the frequency of male skin rejection by the females of the C57BL (subline 1) strain, it was observed that the male skin isograft loss by the young female group required a significantly longer time than that required by the older females.

As an explanation for these observations it might be assumed that the immunological capacity of young females of the A strain is not fully developed due to age limitations. As a result, these recipients failed to reject the male donor tissue. Differences in relative frequency of male skin isograft rejection by the females of both strains may be ascribed to variation in degree of histocompatibility differences present in the A and C57BL (subline 1) strains. The observation that young females of both strains do not reject the male skin isograft as frequently as do older females suggests that the mechanism may reflect a gradual immunological maturation coupled with constantly changing skin properties associated with age. Slow circulatory establishment or the thick and poorly vascularized skin of older animals may contribute to the vulnerability of the isografts from older animals to weaker immunological reactions.

The provocative results obtained in these age studies influenced us to determine the following: (1) whether it might be possible to induce acquired immunological tolerance in female mice of the C57BL (subline 1) and A inbred strains by the intravenous injections of male viable spleen cells during the postneonatal period, and (2) whether induction of tolerance to male skin isograft could be produced in females of the A and C57BL (subline 1) strains by placing them into parabiotic union with isologous males.[16]

TABLE 4

INCIDENCE OF MALE SKIN ISOGRAFT REJECTION IN YOUNG AND OLD FEMALE MICE OF THE C57BL (SUBLINE 1) STRAIN

Strain	Recipient female and donor male age range (days)	Days of observation following skin grafts									
		8-day	Per cent	13-day	Per cent	18-day	Per cent	23-day	Per cent	33-day	Per cent
C57BL (subline 1)	29–34	4/16*	25	5/16	31	11/16	69	11/16	69	13/16	81
C57BL (subline 1)	180	21/24*	87	21/24	87	21/24	87	21/24	87	21/24	87

* Number of female mice showing complete sloughing of the male isograft over total number of mice grafted.

The results of the first experiment for the induction of tolerance by the intravenous injection of male viable spleen cells are shown in TABLE 5. Spleen cell suspensions were prepared according to the method previously described,[15,17] and cell viability was determined by the eosin method.[18]

As newborns, the A and C57BL (subline 1) females between 0 to 24 hours of age received approximately 2 to 4 million adult male viable spleen cells suspended in 0.05 cc. Locke-Ringer's saline solution.

The 30- to 47-day-old A females and the 32- to 48-day-old C57BL (subline 1) females were injected intravenously with approximately 20 million male viable spleen cells in 0.2 cc. Locke-Ringer's solution, and both groups were grafted with prospective male skin isografts at 60 to 72 days and 60 to 61 days, respectively. The 92-day-old A females and 69- to 91-day-old C57BL (subline 1) females were grafted with the male skin isograft at 110 days.

These results indicate that by intravenous injection of adult male viable spleen cells immunological histoincompatibility to male skin isografts exhibited by females of the A and C57BL (subline 1) strains was overcome in the neonates and in older female mice. It is also significant that male skin isotransplant acceptance was approximately the same in females of the A strain injected with spleen cells at either 30 to 47 days, 92 days, or as neonates (87, 92, or 96 per cent, respectively). Female mice of the C57BL (subline 1) strain demonstrated a similar skin isotransplant relationship (100, 80, and 93 per cent).

Also illustrated in TABLE 5 are recent observations that indicate that induction of immunological tolerance can no longer be considered a phenomenon strictly limited to the neonatal period. Following injection of approximately 40 million male viable spleen cells in 0.4 cc. Locke-Ringer's solution, the 178- to 225-day-old A females and the 202- to 212-day-old C57BL (subline 1) females were grafted with male skin isografts at 198 to 244 days and 223 to 233 days, respectively. Both strains accepted the skin isotransplants in every instance.

TABLE 5

INDUCTION OF ACQUIRED TOLERANCE IN FEMALE A AND C57BL (SUBLINE 1) STRAINS FOLLOWING INTRAVENOUS INJECTION OF VIABLE MALE SPLEEN CELLS

Strain	Age when injected c̄ ♂ spleen cells	Age when grafted c̄ ♂ skin (days)	Successful grafts	
			No. takes/total no. grafted	Per cent
A	0–24 hours*	20–34	23/24	96
C57BL (subline 1)	0–24 hours	30–50	28/30	93
A	30–47 days†	60–72	20/23	87
A	92 days	110	12/13	92
A	178–225 days‡	198–244	8/8	100
C57BL (subline 1)	32–48 days†	60–61	9/9	100
C57BL (subline 1)	69–91 days†	110	8/10	80
C57BL (subline 1)	202–212 days‡	223–233	3/3	100
A	Noninjected	29–62	23/40	58
C57BL (subline 1)	Noninjected	29–70	3/35	9

* Approximately 2 to 4 million viable male spleen cells injected per mouse.
† Approximately 20 million viable male spleen cells injected per mouse.
‡ Approximately 40 million viable male spleen cells injected per mouse.

FIGURE 1 shows one of these old A females having accepted a skin isograft from an old A male donor.

The implications of these findings are that both the age limitation and the variations of histocompatibility differences must influence and permit induction of tolerance to male skin isografts.

The next series of experiments involved coelomic parabiosis between male with female and female with female members of both A and C57BL (subline 1) strains. The parabiotic procedure used was a modification of those of Sauerbruch and Heyde[19] and Bunster and Meyer.[20] Hair was clipped from the opposite lateral sides of each pair. An incision, including skin and muscles, was then made from the ear base to the proximal insertion of the femur. Confluence of the coelomic cavities was achieved by extending the incision into the peritoneal cavity from the lower edge of the last rib to the iliac crest. The peritoneums of both animals were joined and closed, using No. 5-0 plain catgut continuous sutures. The skin and muscles of the dorsolateral and ventro-

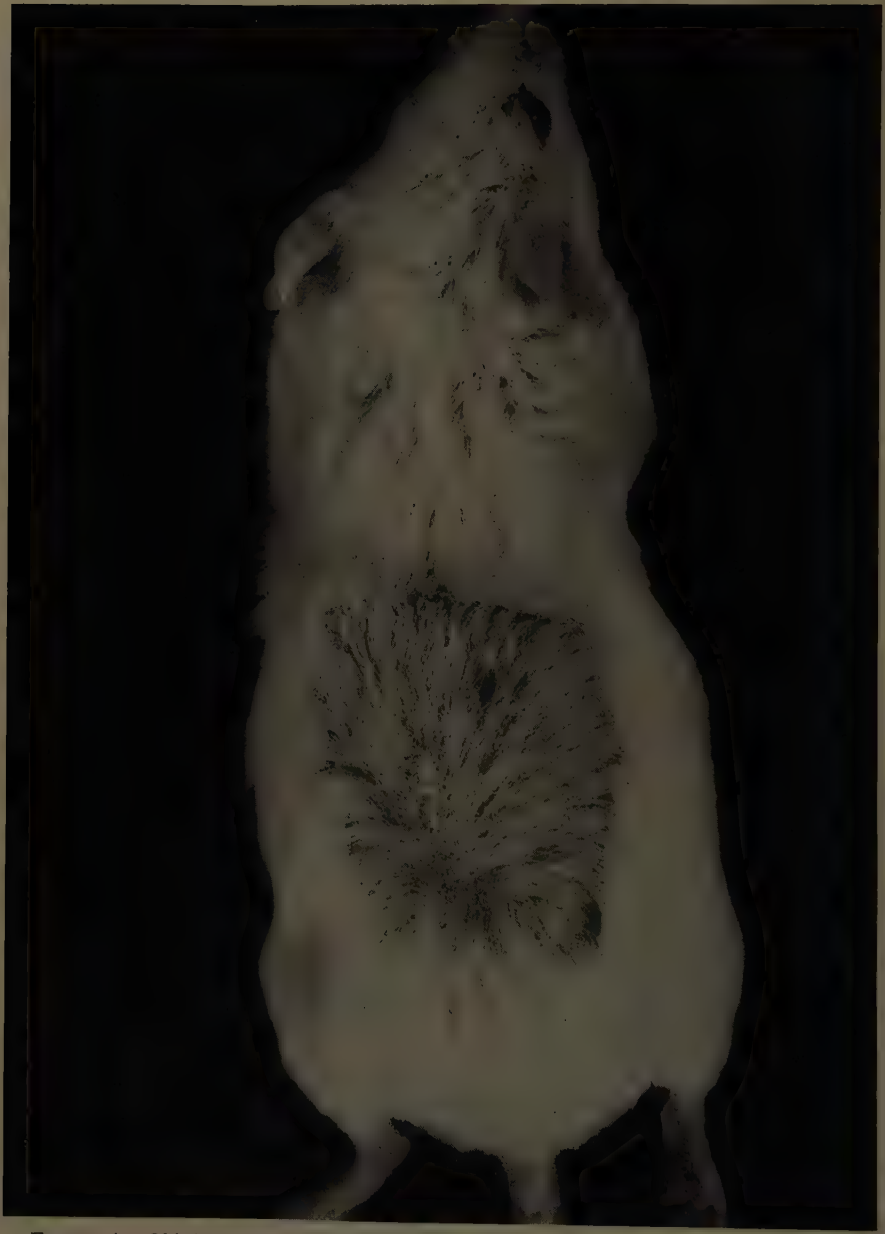

FIGURE 1. Old A♀ with an old A♂ skin graft. A♀ had received, prior to transplantation, an intravenous injection of male spleen cells.

lateral flaps were closed with Michel skin clips, which were removed after 7 days. No bandage or dressing was used. Skin isografts were exchanged between the respective members of each parabiotic pair following establishment of parabiosis. The procedure for skin transplants and the length of observation periods were similar to those previously described.

The control groups of female to female parabionts were grafted with male skin isotransplants from donors of appropriate ages.

TABLE 6 shows the ages of the parabionts, time span for parabiotic establishment prior to skin grafting, and the results from this study. The male skin isograft acceptance was 90 per cent in the A strain and 87 per cent for C57BL (subline 1) strain. None of these control groups of either strain accepted the male skin isotransplant. FIGURES 2 and 3 show a successful male skin isograft on a female in parabiosis with an isologous male in the A and C57BL (subline 1) strains, respectively. Other recent observations indicate that the integrity of the parabiotic union need not be maintained to facilitate the subsequent successful isotransplants. Furthermore, tolerance may be transferred between tolerant females placed in parabiosis with normal unconditioned females.

TABLE 6

INDUCTION OF ACQUIRED TOLERANCE IN FEMALE A AND C57BL (SUBLINE 1) STRAINS TO MALE SKIN ISOGRAFTS FOLLOWING PARABIOSIS

Parabiosis groups	Age when joined in parabiosis (days)		Time in parabiosis (days)	Female parabionts accepting male skin graft* No.	Per cent
	♀	♂			
A♂ ↔ A♀	58–93	55–127	7–14	19/21	90
C57BL♂ ↔ C57BL♀	53–101	51–72	9–12	13/15	87
A♀ ↔ A♀	60	—	14–15	0/5	0
C57BL♀ ↔ C57BL♀	62–104	—	9–15	0/9	0

* No. takes/total no. grafted.

TABLE 7 shows that there was a 100 per cent acceptance of male skin isografts by the females of C57BL (subline 1) strain when left in parabiosis for periods as short as 4 days. Full-thickness skin isografts were exchanged between the members of each parabiotic pair several days following the interruption of the parabiotic union.

TABLE 8 indicates that tolerance was induced in normal unconditioned adult female mice of the C57BL (subline 1) strains when they were placed in parabiotic union with C57BL (subline 1) tolerant females that had received an intravenous injection of male viable spleen cells at 32 to 48 days of age. In every instance the normal female accepted the male skin isograft following the establishment of parabiosis. These results indicate that antigens, presumably present as intact male cells, circulate and are exchanged in sufficient numbers between the parabionts, and this results in induction of tolerance to male skin isografts in the unconditioned female recipient.

In recently published findings by Martinez *et al.*[21] further evidence has been presented that in certain limited but well-defined systems tolerance may be

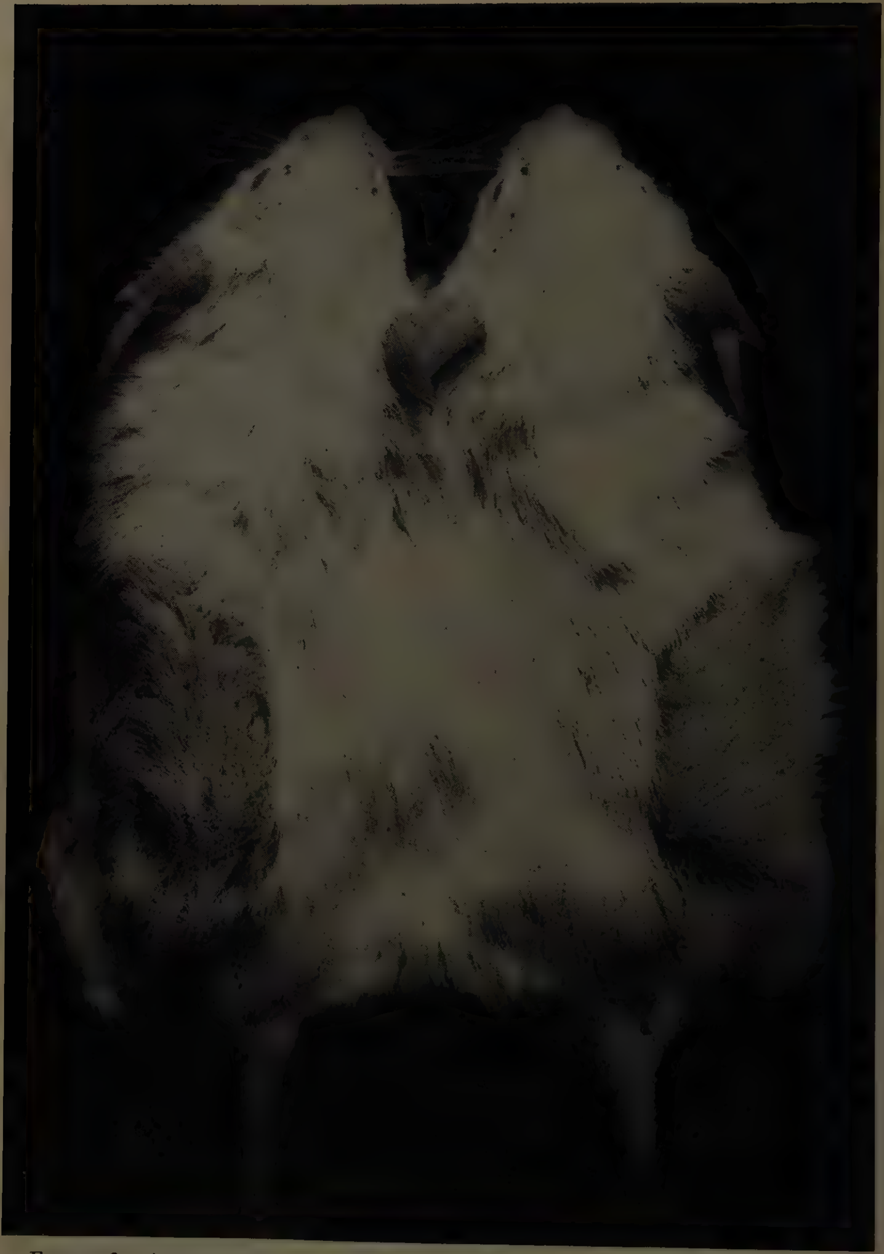

FIGURE 2. A♂ ↔ A♀ parabiosis. Left animal is A♀ with A♂ skin graft. Right animal is A♂ with A♀ skin graft.

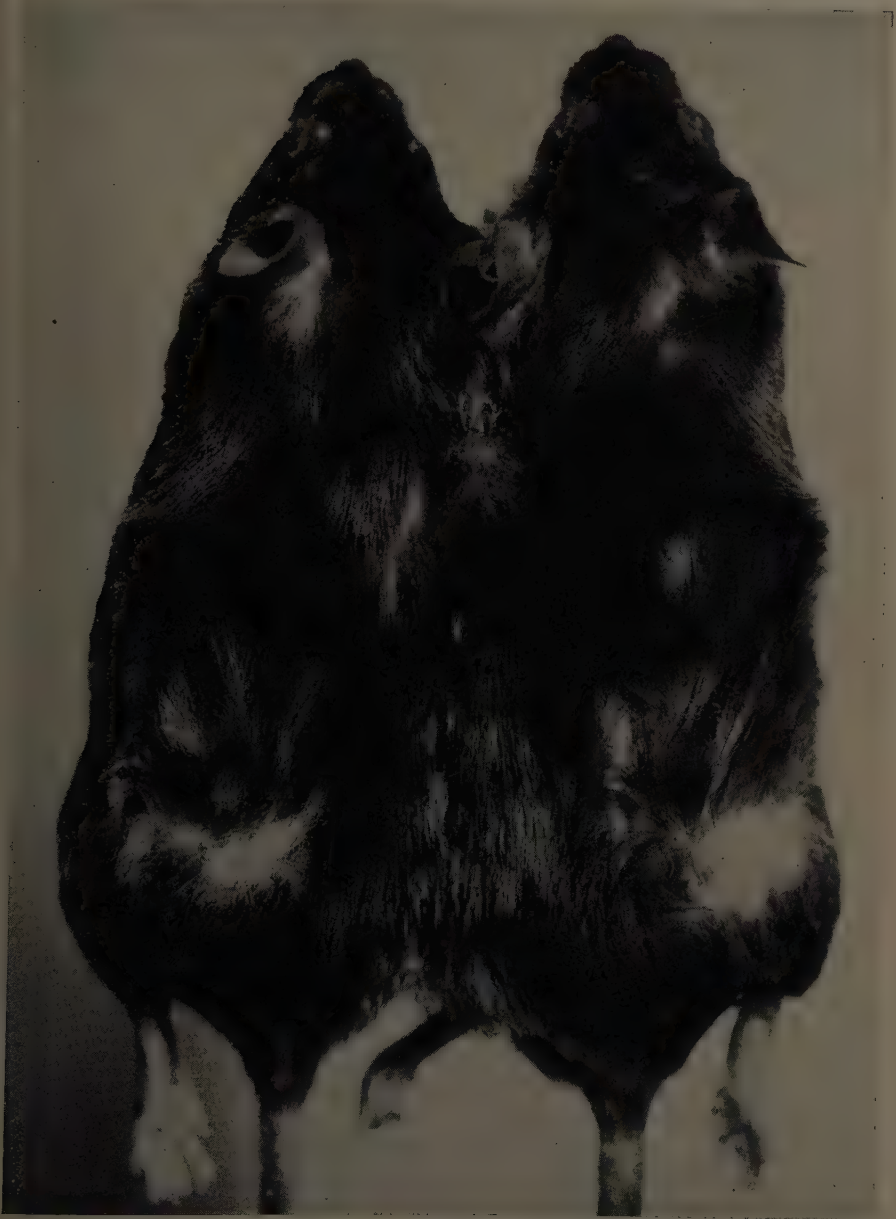

FIGURE 3. C57BL (subline 1) ♂ ↔ C57BL (subline 1) ♀ parabiosis. Left animal is C57BL (subline 1) ♀ with C57 (subline 1) ♂ skin graft. Right animal is C57 (subline 1) ♂ with C57BL (subline 1) ♀ skin graft.

produced in older animals by intravenous injection of viable spleen cells or by establishment of parabiosis. It has been found further that, in these instances as well, tolerance may be transferred by the establishment of a parabiotic union. Production of immunological tolerance through embryonal parabiosis was an earlier observation of Hasek and Hraba,[22] but the burden rests on our observations that parabiosis in postneonatal periods may permit the establishment of immunological tolerance.

TABLE 7

INDUCTION OF ACQUIRED TOLERANCE IN ADULT FEMALE MICE OF THE C57BL (SUBLINE 1) STRAIN BY PARABIOSIS

Group	No. of pairs	Time in parabiosis (days)	Age of parabionts (days)	Parabionts accepting male skin grafts	
				No.	Per cent
♂ ↔ ♀	14	4–8	67–125	14	100
♀ ↔ ♀	9	9–15	62–104	0	0

TABLE 8

TRANSFER OF ACQUIRED IMMUNOLOGICAL TOLERANCE TO MALE SKIN ISOGRAFTS IN C57BL (SUBLINE 1) FEMALE MICE BY PARABIOSIS

Parabiosis	No. of pairs	Age of parabionts (days)	Time in parabiosis (days)	No. of pretreated parabionts accepting male skin grafts	No. of non-pretreated parabionts accepting male skin grafts
♀ Tolerant of male* × normal ♀	5	116–177	13	5	5
Normal ♀ × normal ♀	9	62–104	9–15	—	0

* Tolerance was induced by intravenous injection of approximately 20 million viable male spleen cells in postneonatal period (32–48 days of age).

The findings that we have presented are difficult to interpret according to current conceptual understanding of immunological tolerance. However, the four following conclusions are evident:

(1) The Eichwald-Silmser phenomenon is further substantiated.

(2) The histocompatibility barrier between males and females of specific inbred strains can be overcome by experimental procedures designed to produce classic immunological tolerance.

(3) That induction of immunological tolerance no longer may be viewed as a phenomenon limited to the neonatal period. The relatively weak histocompatibility differences between males and females of the A and C57BL (subline 1) strains can be overcome at any age either by the intravenous injections of male viable spleen cells or by placing the males and females in parabiotic union.

(4) In the females of both A and C57BL (subline 1) inbred strains the immunological unresponsiveness to male skin isografts appears to be permanent

and indistinguishable, whether tolerance is produced by either spleen injection or parabiosis during the neonatal or postneonatal period.

The data presented seem to provoke further consideration concerning the essential relationship between immunological tolerance and immunological paralysis,[23] immunological enhancement,[24] specific immunological unresponsiveness in adults,[25] specific immunological unresponsiveness facilitated in adult animals by irradiation,[26,27] and other inhibitors of the immune response.[28]

References

1. EICHWALD, E. & C. R. SILMSER. 1955. Transplantation Bull. **2:** 148.
2. PREHN, R. T. & J. MAIN. 1956. J. Natl. Cancer Inst. **17:** 35.
3. SHORT, B. F. & W. R. SOBEY. 1957. Transplantation Bull. **4:** 110.
4. BILLINGHAM, R. E. & W. K. SILVERS. 1958. Science. **128**(3327): 780.
5. MARIANI, T., C. MARTINEZ, J. M. SMITH & R. A. GOOD. 1958. Proc. Soc. Exptl. Biol. Med. **99:** 287.
6. SACHS, L. & E. HELLER. 1958. J. Natl. Cancer Inst. **20:** 555.
7. BERNSTEIN, S. E., A. A. SILVERS & W. K. SILVERS. 1958. J. Natl. Cancer Inst. **20:** 577.
8. ZAALBERG, O. B. 1959. Transplantation Bull. **6:** 433.
9. EICHWALD, E. J., C. R. SILMSER & N. WHEELER. 1957. Ann. N. Y. Acad. Sci. **64**(5): 737.
10. HAUSCHKA, T. S. 1955. Transplantation Bull. **2:** 154.
11. SNELL, G. D. 1956. Transplantation Bull. **3:** 29.
12. EICHWALD, E. J., C. R. SILMSER & I. WEISSMAN. 1958. J. Natl. Cancer Inst. **20:** 563.
13. MARIANI, T., J. M. SMITH, R. A. GOOD & C. MARTINEZ. 1959. Am. Assoc. Cancer Research. **3:** 40.
14. MARIANI, T., C. MARTINEZ, J. M. SMITH & R. A. GOOD. 1959. Proc. Soc. Exptl. Biol. Med. **102:** 751.
15. MARTINEZ, C., J. M. SMITH, J. B. AUST & R. A. GOOD. 1957. Proc. Soc. Exptl. Biol. Med. **98:** 736.
16. MARIANI, T., C. MARTINEZ, J. M. SMITH & R. A. GOOD. 1959. Proc. Soc. Exptl. Biol. Med. **101:** 596.
17. BILLINGHAM, R. E. & L. BRENT. 1957. Ann. N. Y. Acad. Sci. **69**(4): 678.
18. SCHREK, R. & F. W. PRESTON. 1957. Cancer Research. **17:** 102.
19. SAUERBRUCH, F. & M. HEYDE. 1908. Münch. med. Wochschr. **55:** 153.
20. BUNSTER, E. & R. K. MEYER. 1933. Anat. Record. **57:** 339.
21. MARTINEZ, C., J. M. SMITH, F. SHAPIRO & R. A. GOOD. 1959. Proc. Soc. Exptl. Biol. Med. **102:** 413.
22. HASEK, M. & T. HRABA. 1955. Nature. **175:** 764.
23. FELTON, L. D. 1949. J. Immunol. **61:** 107.
24. KALISS, N. 1955. Ann. N. Y. Acad. Sci. **59**(3): 385.
25. JOHNSON, A. G., D. W. WATSON & W. J. CROMARTIE. 1955. Proc. Soc. Exptl. Biol. Med. **88:** 421.
26. DIXON, F. J. & P. H. MAURER. 1955. J. Exptl. Med. **101:** 245.
27. TRENTIN, J. J. 1957. Proc. Soc. Exptl. Biol. Med. **96:** 139.
28. SCHWARTZ, R. 1959. J. Clin. Invest. **38:** 1041.

Discussion of the Paper

E. C. LUSTGRAAF: I find the observations of Mariani very interesting primarily because they agree so well with those we have obtained and with those that Billingham has reported.

Probably the only obvious method of inducing tolerance that has not been mentioned is irradiation of the female followed by the injection of pertinent male cells. Needless to say, this method has worked very well.

We also find that in our BALB/c mice, contrary to their findings, the adult female, in about 80 per cent of the trials, will reject an isologous male graft.

We have been able to render the vast majority of the females tolerant by the intraperitoneal injection of large doses of male spleen cells some time prior to grafting.

Our results in parabiosis have not been nearly as favorable or universal as Mariani has reported. I think possibly it may be that we have consistently separated our animals at the time of grafting or at the time when we are ready to graft them. Furthermore, we have never exchanged grafts between partners; instead, we have used "stock" male animals as donors.

Perhaps the most interesting and probably the most important of the observations we have made regarding the tolerance of male tissue by the female is what we call, for lack of a better term, "split tolerance." The specificity of the male antigen does not seem to exist.

I think Billingham has also observed the phenomenon that we call split tolerance. When you take a newborn female mouse of one strain and inject it with male spleen cells from a different strain, she will become tolerant of the maleness of these cells, as if she had been injected with isologous male spleen cells, but she will not become tolerant of the strain itself which served as donor for the cells in the pretreatment. This split tolerance has brought to our mind the question as to whether the donor cells actually do persist and, if so, why they should induce tolerance to their maleness but not to their strain. Is there really any need that the cells persist or survive in order to induce tolerance?

We have also wondered whether the number of cells needed to induce strain tolerance is perhaps much greater than that needed for the induction of sex tolerance.

SILVERS: I may say that we have done similar work, and that we get a completely different picture. We find that all C57 females injected at birth with male cells are tolerant. We find that the tolerance-responsive period lasts to about 17 days of age, at which time about 60 per cent of injected females become tolerant. Thereafter, after 20 days of age, we do not get tolerance; in fact, we get immunity. Furthermore, we are able to abolish tolerance of the Y factor at any time by injecting isologous immune lymph-node cells. So, I wonder what we are doing differently from what the Minnesota group is doing? Our cell dosage to obtain tolerance in 17-day-old females is in the neighborhood of about 12 to 15 million spleen cells.

I also think that this concept of split-tolerance does give good evidence that tolerance can persist without the persistence of the cells that have brought the state of tolerance into being. I say this because we have injected C57 females with *A* male cells; when these C57 females grew up, they were challenged with an *A* skin graft, which they promptly rejected. Thereupon they were challenged with a C57 male isograft, which they accepted indefinitely, even though the period of time between the original inoculation of the male spleen cells and the test grafting was 120 days. Moreover, if we assume that the Y antigen is able to persist in the absence of the cells in which it was introduced, then we might expect that, in those animals that are tolerant but not cellular chimeras, we should be able to transfer tolerance by taking spleen cells from these females and inoculating them into other newborn female C57s. However, we have not been able to do this. This indicates that, if the Y antigen does persist after the cells in which it was introduced are rejected, it cannot persist in great quantities.

EICHWALD: You do not think that is due to dilution?

SILVERS: I can say only that we find that as few as 10,000 isologous male leukocytes injected into newborn females will render some tolerant, so that the number of cells necessary to produce tolerance to the Y factor is not very great.

MARTINEZ: In answer to part of Silver's question about what we in Minnesota are doing that is different from what they are doing in Philadelphia: in the first place, perhaps there is some difference in the genetic make-up of the animals that they are using as compared to those that we are employing. I refer especially to the C57BL mice which, as you may notice, we have used in the subline 1. I believe that Billingham and Silvers have used primarily the subline 6. This might account for differences observed by our two groups.

SILVERS: We have also tried transferring tolerance with parabiosis. We have taken *A* animals injected at birth with *CBA* cells that were indeed tolerant when test-grafted. We have parabiosed these tolerant animals with normal *A* animals. We find that in every case the graft on the tolerant animal is rejected. I think that you have reported just the opposite—that the normal animal becomes tolerant?

MARTINEZ: It depends upon the different strains of the host and donor exchange combinations that are used: the degree of histocompatibility difference between the host and the donor that is the factor chiefly responsible for the induction or transfer of acquired tolerance in mice.

EICHWALD: You don't think graft size is relevant?

MARTINEZ: It might be, but I cannot say so from my own experience. We have been using, most of the time, a graft that is 1½ centimeters square.

EICHWALD: Yours are even smaller, are they not?

SILVERS: Yes, ours are smaller, 1 centimeter square, perhaps.

PREHN: Some years ago, when we studied the Eichwald-Silmser effect, we did not demonstrate it in our BALB/cAn mice, even though second-set grafts were applied. I think it was Eichwald who, some time afterward, said that probably we had not observed our grafts long enough during the postoperative period or undoubtedly we should have seen that they were ultimately rejected. Therefore the question is: How long did Mariani observe grafts postoperatively in BALB/c mice?

MARIANI: For more than one year. In fact, we purposefully sacrificed them all about the first part of September, 1959. The procedure in our laboratory is to observe them from six months to a year postoperatively; then we must sacrifice them because of local statutes.

STRENGTH OF HISTOCOMPATIBILITY GENES*

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Histocompatibility genes may be ranked according to their relative effectiveness in curtailing the survival of homografts; strong genes are defined as those causing prompt immune responses, weaker ones as those permitting longer graft acceptance on incompatible hosts. Formal demonstration of the strength of individual genes requires donors and recipients that are identical except for the alleles in question, that is, members of the coisogenic lines of mice developed by Snell.[1] The strains C57BL/10, B10.D2, and B10.LP, as described by Counce and her associates,[2] offer combinations that differ only at the strong H-2 and/or the weaker H-3 locus. In TABLE 1 are shown the differential alleles setting these lines apart and the survival times of skin homografts exchanged between them. The question that has concerned us in the studies to be summarized is: Why are the effects of these genes so varied or, in more pointed terms, what are the differences in the quality or quantity of the corresponding transplantation antigens that account for the observed differences in survival time?

Methods and Materials

Skin homografts and survival times. The method of grafting and the criteria for evaluating the survival of skin homografts were identical to those described by Billingham and Medawar.[3] Only donors in an inactive phase of the hair growth cycle were used. Sex-linked incompatibility was avoided by using donors and recipients of the same sex. The age of the animals was between 8 and 12 weeks.

The survival data, shown in TABLE 1, the distribution of first- and second-set rejection, and the behavior of skin homografts exchanged between the strains have been discussed previously.[4] The conclusions we shall draw presently are based on the following observations: (1) the median survival time of the first-set skin homografts exchanged across the H-2 locus is 9 days; but (2) such grafts are not destroyed perceptibly by day 6 in the absence of prior immunization; (3) first-set skin homografts exchanged across H-3 incompatibility ($H\text{-}3^a \rightarrow H\text{-}3^b$) are never affected before day 16 of residence in the absence of prior immunity; and (4) second-set homografts in that combination are fully destroyed by day 8 on animals that have received and rejected a previous first-set graft. In each case the degree of destruction was based on the amount of surviving epithelium, as determined visually upon gentle removal of the epidermal "ghost" and subsequent exposure to drying at inspection.

Genetic composition of mouse strains.† The origin and development of the coisogenic lines used here has been described by Snell.[1] Although the strains should have reached, in theory, a high degree of purity and approach coisogenicity in all significant respects except those of the genetic constitutions indicated in TABLE 1, confirmation of certain details of their composition was made, using skin homografts as the criterion.[3] It has been established that each of the lines is isogenic; that is, isografts exchanged between members of the same lines are now surviving past 200 days. The proportion of parental strain homografts now surviving past 100 days on the F$_2$ progeny of crosses between C57BL/10 and B10.D2, and past 50 days on the F$_2$ of crosses between C57BL/10 and B10.LP does not deviate significantly from the ratio 3:4 that indicates the difference of only a single gene between the parents. If any additional weaker gene differences than those previously tabulated were present, they would not complicate the results of experiments involving durations of less than 50 days.

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* The opinions and assertions contained in this paper are those of the authors and are not to be construed as official, nor as reflecting the views of the Navy Department or of the naval service at large.

† Breeding stocks of the mouse strains used in this study were generously provided by George D. Snell, Roscoe B. Jackson Memorial Laboratory, Bar Harbor, Maine.

TABLE 1

Strain	Differential alleles	
	H-2 locus	*H-3* locus
C57BL/10ScSn	b	a
B10.D2/Sn	d	a
B10.LP/Sn	b	b

Survival times of skin homografts (days)

Combination	First-set (median)	Second-set (all grafts destroyed)
C57BL/10 ↔ B10.D2	9	6
C57BL/10 → B10.LP	20	8
B10.LP → C57BL/10	34	8

Tests were carried out to confirm that the strains C57BL/10 and B10.D2 share alleles at the H-3 locus (H-3^{a}) that the strain B10.LP (H-3^{b}) is lacking. Provided that the strains are composed as indicated in TABLE 1, B10.LP recipients, after having rejected primary skin homografts from C57BL/10 donors, should subsequently reject second-set skin homografts from B10.D2 donors, on the basis of a reaction against H-3^{a} antigens. Eighteen C57BL/10-immune B10.LP recipients were tested. Because only 9/18 of the grafts were destroyed by day 6, H-2 incompatibility was excluded as the basis. Since all grafts (18/18) were fully destroyed by day 8, it was concluded that anti-H-3^{a} immunity is responsible, and that the suggested H-3 genotypes are correct.

Experimental Results

Antigenic activity of spleen cells in H-3 incompatibility. Previous investigations involving strongly incompatible strain combinations have led to the opinion that spleen cells have the same antigenic complement as have other tissues, especially skin, and exhibit all of the transplantation antigens common to the donor strain.[5] This hypothesis is founded on observations that spleen cells are capable of immunizing recipients of one strain against skin homografts from another and of inducing tolerance of later skin homografts upon injection into the recipients immediately postpartum.

An observation that initially appeared to contradict the hypothesis of equivalence was our finding that spleen cells are totally inactive when H-3 incompatibility alone is involved (C57BL/10 → B10.LP), a result that applied to a wide range of dosages (5 to 50 million cells) injected intraperitoneally over times ranging from 21 days prior to test grafting to 18 days after the test grafts were placed on their recipients. In contrast, spleen cells are highly potent, immunizing agents when H-2 incompatibility is involved, and the reaction against them is far more rapid than that against a first-set orthotopic skin homograft, as determined by the time of onset of second-set reactivity.[6]

On further investigation it was found that the observed inactivity of spleen cells was confined to the special case of inoculation by the intraperitoneal route. Although it has been shown previously that the route of injection is of little importance when strong incompatibility is present,[7] it was found to be of decisive significance when H-3 incompatibility alone was involved. Upon subcutaneous or intravenous injection of C57BL.10 spleen cells into B10.LP recipients, some marginal immunity was promoted; of 19 grafts, 10 were rejected by day 13. Thus spleen cells of H-3^a-bearing mice contain the complement of antigens associated with this allele, a fact that was substantiated by finding that these cells induce tolerance in newborn B10.LP hosts. Of 18 B10.LP recipients injected with 5 million C57BL.10 spleen cells intravenously within 24 hours after parturition, all were fully tolerant of later test grafts for more than 100 days.

The results of these initial experiments, therefore, were far from what might have been expected on the basis of previous experiences with strongly incompatible strain combinations. On the other hand, feeble immunization obtained under the best of circumstances by the subcutaneous route accorded with what might have been anticipated if the weakness of H-3 alleles is but a reflection of insufficient antigen within the tissues. The first evidence that the explanation might not be so elementary came when the antigenic effectiveness of C57BL.10 dissociated epidermal cells was tested in B10.LP hosts. The observation that 3 to 4 million epidermal cells injected 8 days before test grafting rendered their recipients so highly immune that the test grafts were rejected fully within 6 days (6/6) gave rise to the suspicion that it was not the concentration of antigens within the cells that limited their effectiveness as immunizing agents as much as the failure of antigens to make the required contact with their hosts.

Cell-free antigenic extracts of spleen cells. Might not the inactivity of spleen cells injected intraperitoneally reside in the failure of the cells to liberate H-3 transplantation antigens? Using our routine version[8] of the Billingham, Brent, and Medawar procedure[9] for preparing cell-free antigenic extracts of spleen cells, the antigens yielded by 250 mg. of spleen (wet weight) rarely fail to induce strong immunity in recipients that differ from the spleen donors by H-2 incompatibility. It is therefore noteworthy that repeated attempts to induce immunity of B10.LP hosts to C57BL/10 splenic extracts have failed, even when the dosage was increased to 500 mg. per recipient, the equivalent of 4 to 5 spleens. Such a result suggests another aspect of the distinction between H-2 and H-3 antigens, the latter not being extractable by the same method that has proved so effective for the H-2 antigens (*cf.* Berrian and Jacobs[8]). Differences in the physical attachments of the antigens within the cells or in the chemical properties that affect their behavior upon extraction might account for the findings. As the experiments next to be described show, it is quite unlikely that the failure to obtain H-3^a antigens from spleen cells is explained by a low concentration within the cells since, under the circumstances to be described, spleen cells are nearly as active in inducing immunity against H-3 antigens as against those of H-2.

H-3 activity of spleen cells in the presence of H-2 incompatibility. The effectiveness of spleen cells as immunizing agents when H-2 or another strong incompatibility is involved has been attributed to their sequestration within the hosts' lymphoid centers.[10] If such a fate is required for immunization and is ensured by strong antigenic differences, would H-3 antigens present in the cells also share in the recognition accorded H-2 antigens? The mouse strains offered a test of this hypothesis, since mice of strain B10.D2 differ from the B10.LP line at both H-2 and H-3, and the former has the H-3^a allele whose activity is in question. Consequently a number of experiments were carried out to determine whether B10.D2 spleen cells, upon intraperitoneal injection into B10.LP hosts, incite immunity against H-3^a antigens as well as those of H-2^d. In testing with C57BL/10 homografts, that differ from the recipients only at H-3, only the immunity directed against the H-3^a antigens was challenged.

The results leave little doubt that, in fact, the additional provision of H-2 incompatibility leads to a prompt response against the H-3^a antigens of spleen cells. Of 40 recipients tested in this manner, 37 rejected C57BL/10 skin homografts by day 8. Furthermore, second-set responses were obtained regularly when the interval between injection and test grafting was reduced to 4 days. By comparison, about 8 days' preimmunization with skin homografts is required to bring about the same effect. The minimal time required to immunize recipients against the H-3^a antigens in the presence of H-2 incompatibility is thus reduced significantly.

Spleen cells then were tested in the same manner, by the subcutaneous route of injection, to determine whether the augmentation of H-3 immunity by the presence of H-2 incompatibility improved the feeble responses promoted in the face of H-3 incompatibility alone. Following the injection of 50 million C57BL/10 spleen cells into 19 B10.LP recipients, all of the test grafts survived past day 8, although they were rejected well before the normal first-set MST (median survival time). By comparison, complete immunization was obtained with B10.D2 spleen cells, and 10 of 11 B10.LP recipients rejected their C57BL/10 test grafts by day 8 of residence. The presence of H-2 incompatibility thus accelerates the development of immunity to H-3^a antigens by the subcutaneous as well as the intraperitoneal route.

Independence of H-2 and H-3 immunization by skin homografts. When skin homografts from C57BL/10 donors were placed together with B10.D2 strain grafts on the same B10.LP recipients, the grafts of each kind were found to survive to their normal first-set MST. Thus the combined presence of H-2 and H-3 antigens in the one graft (B10.D2) does not augment the rate at which the more slowly arising H-3 immunity develops. The results may be explained by the assumption that *skin homografts* immunize their recipients through the elaboration of free antigenic molecules rather than by the transfer of intact cells or subcellular particles serving as the common vehicle for the two different antigens.

Discussion

When genes are defined in terms of their relative effectiveness through the survival of first-set skin homografts, the differences are clear, at least as long as the donors and recipients differ only in so far as the genes in question. Such distinctions, which are evident in coisogenic situations, may hold up even in the presence of additional incompatibility. Our experiments on the latter point show that, as long as the source of antigens is an orthotopic skin homograft, there may be little or no interference between antigens, immunity to each of them arising at *independent rates*. However, when spleen cells and, by inference, other injected cells are put to the same test, the presence of one kind of antigen may affect the immunity arising against another, and the distinctions in the strength of histocompatibility genes under these circumstances may lose their meaning.

Some additional implications from the studies described here may be stated. First, spleen cells are not the equivalent of skin homografts in respect of their ability to induce immunity. Although spleen cells under certain circumstances are highly effective as immunizing agents, they may be totally inactive in other situations or only feebly active, depending on the route of immunization. Second, in the presence of multiple histocompatibility differences, spleen cells may procure a broader spectrum of immunity against the weaker antigens than do skin homografts, a possibility that must affect any analysis based on the concept that the two tissues promote an equivalent response. This appears to be a consequence of the ability of one antigen to trade on the recognition afforded foreign cells by the presence of another, a commensal relationship that is not exhibited in the case of the orthotopic skin graft. Finally, the strength of transplantation antigens or, conversely, their weakness does not appear to be a consequence of the concentration of antigens as much as to reflect their distribution within the cells and their accessibility to host recognition.

References

1. Snell, G. D. 1958. Histocompatibility genes of the mouse. II. Production and analysis of isogenic resistant lines. J. Natl. Cancer Inst. **21:** 843.
2. Counce, S., P. Smith, R. Barth & G. D. Snell. 1956. Strong and weak histocompatibility gene differences in mice and their role in the rejection of homografts of tumors and skin. Ann. Surg. **144:** 198.
3. Billingham, R. E. & P. B. Medawar. 1951. The technique of free skin grafting in mammals. J. Exptl. Biol. **28:** 385.
4. Berrian, J. H. & C. F. McKhann. 1960. Transplantation immunity involving the H-3 locus. J. Natl. Cancer Inst. In press.
5. Medawar, P. B. 1959. Iso-antigens. In Biological Problems of Grafting. Congr. Coll. Univ. Liége. **12:** 6.
6. McKhann, C. F. & J. H. Berrian. 1959. Transplantation immunity: some properties of induction and expression. Ann. Surg. **150:** 1025.
7. Billingham, R. E., L. Brent & N. A. Mitchison. 1957. The route of immunization in transplantation immunity. Brit. J. Exptl. Pathol. **38:** 467.

8. Berrian, J. H. & R. L. Jacobs. 1959. Diversity of transplantation antigens in mice. In Biological Problems of Grafting. Congr. Coll. Univ. Liège. **12:** 131.
9. Billingham, R. E., L. Brent & P. B. Medawar. 1956. The antigenic stimulus in transplantation immunity. Nature. **178:** 514.
10. Mitchison, N. A. 1956. The colonisation of irradiated tissue by transplanted spleen cells. Brit. J. Exptl. Pathol. **37:** 239.

A STRAIN-SPECIFIC INFLUENCE OF PARITY ON RESISTANCE TO HOMOGRAFTS*

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The homograft problem is often looked upon from a theoretical standpoint as somewhat of an artifact or laboratory curiosity, although its practical aspects are forced upon the attention of clinicians. The question may be asked, "What purpose is served by transplantation antigens and antibodies in nature?" In our speculations we have adopted the viewpoint that what we call antigens, antibodies, and immunity in this special context are probably elements of a biological information system of broad application and that they might carry other names had we the knowledge to assign them.

Gestation in mammals is one naturally occurring phenomenon that seems related to the subject. In randomly bred populations the fetus and mother have genetic relationships similar to those between F_1 hybrids and parental strains, if differences between homozygous and heterozygous populations are temporarily disregarded. A hybrid graft is not expected to grow in the maternal strain but, apparently, the mammalian fetus is an exception. Probably many factors are operative, but we wondered if some other evidence of "tolerance" in the mother could be observed.† One of the first questions was, "Does the mother acquire some sort of tolerance demonstrable by current techniques?" We have found that she does in that, after females of one inbred strain were mated to males of their own strain or of a second strain, the post-partum females of the latter class exhibited tolerance toward a homologous graft isologous with the second strain. This was seen only in those females previously mated to males genetically related to the graft and not in those mated to males of their own strain or of a third strain unrelated to the graft.

Materials and Methods

All host animals were female BALB/cAnN mice. They were force-bred (remated on the day of parturition), and all litters were discarded. Prior to the experimental procedures outlined below, the females had had no known exposure to the antigens of the male other than, as adults, through the processes of mating, gestation, and parturition. The tumor used for challenge[2] was sarcoma DBA49. This tumor originated as a "spontaneous" tumor in a DBA/2 female, but is transplantable to strain BALB/c and has been maintained in this strain. The tumor grows equally well in both sexes and, during the course of these experiments, it grew progressively in 71 per cent (211/298) of untreated BALB/c mice.

Each female, postpartum or virgin, was inoculated once with a dose of 0.025 ml. of a 50 per cent tumor suspension in saline given as a single subcutaneous inoculation in the right flank. Parous females were inoculated after the birth

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† The term tolerance is used in the general sense of "enhanced acceptance" or "failure to resist or react" rather than in the strict sense defined by Billingham *et al.*[1]

of their last litter. In all but the first experiment, females were immunized with 0.05 ml. of DBA/2 blood 10 days prior to implantation of the tumor. Parous females were immunized within 10 days following the birth of their last litter. Each female received 2 simultaneous subcutaneous inoculations of 0.025 ml. of whole cardiac blood in 2 sites on the right thoracic wall. The blood for each inoculation site was drawn from a separate DBA/2 female. This amount of blood is 2½ times that necessary to produce a high degree of resistance to this tumor in BALB/c mice.[3]

The data in the following experiments represent aggregates of several small experiments, each having concurrent controls.

In this paper the word take refers to the death of a host bearing a progressively growing tumor or, in a small number of instances, to a host bearing a tumor greater than a minimal size of 7 by 7 mm. at 4 weeks, which has been found to be lethal if the animals are observed for a longer period of time.

TABLE 1

EFFECT OF PARITY FOLLOWING INTRA- AND INTERSTRAIN MATINGS ON "NATURAL" RESISTANCE TO TUMOR DBA49 IN BALB/c FEMALES

No. litters	Females previously mated to:*			
	DBA/2 males		BALB/c males	
	Takes/total	Per cent	Takes/total	Per cent
1	34/41	83	41/54	76
2	25/29	86	13/16	81
3 to 6	36/36	100	32/39	82
Totals	95/106	90	86/109	79

* The tumor took in 110/164 (67 per cent) of virgin females.

Results

Three groups of BALB/c females were treated as follows. One group was mated to DBA/2 males, a second group was mated to BALB/c males, and a third group was retained as virgins. Both groups of parous females were bred to produce from 1 to 6 litters and each was inoculated with tumor tissue within 10 days after the birth of its last litter. The results given in TABLE 1 are not very convincing due to the fact that this tumor normally grows in a high percentage of BALB/c mice and that the possible range of difference is small. The DBA/2 tumor took in a higher over-all percentage of females that had borne litters by DBA/2 males than it did of those parous by BALB/c males (90 per cent:79 per cent; $P = 0.035$). An apparently nonspecific effect of previous pregnancy was reflected by a higher percentage of takes in females parous by BALB/c males than in virgin females (79 per cent:67 per cent; $P = 0.037$). However, the tumor took in a significantly higher percentage of females that had borne 3 or more litters by DBA/2 males than of those having had 1 or 2 litters by DBA/2 males (100 per cent:84 per cent; $P = 0.013$). No such increase was seen in females mated to BALB/c males.

The technique of prior immunization was used in a second experiment because it was hoped that by this means the apparent effect would be more pronounced; such was found to be the case. Three groups of BALB/c females were treated as in the previous experiment. One group was mated to BALB/c males and a second to DBA/2 males, to produce from 1 to 6 litters. Parous females were immunized with DBA/2 blood within 10 days after the birth of their last litter. Virgins of approximately the same age also were immunized to serve as controls. Ten days after immunization all 3 groups were challenged with sarcoma DBA49. The results given in TABLE 2 were highly significant in that the tumor took only in those females parous by DBA/2 males (52 per cent) and not in virgins or in females parous by BALB/c males. As in the previous experiment, an apparently cumulative effect was observed: females that had borne 3 or more litters by DBA/2 males showed a significantly higher percentage of tumor takes than did those that had had only 1 or 2 litters (83 per cent:46 per cent; $P < 0.001$).

TABLE 2

EFFECT OF PARITY FOLLOWING INTRA- AND INTERSTRAIN MATINGS ON INDUCED RESISTANCE TO TUMOR DBA49 IN BALB/c FEMALES

No. litters	Females previously mated to:*			
	DBA/2 males		BALB/c males	
	Takes/total	Per cent	Takes/total	Per cent
1	40/93	43	0/61	0
2	12/21	57	0/18	0
3 to 6	20/24	83	0/36	0
Totals	72/138	52	0/115	0

* The tumor did not take in immunized virgin females (0/89).

We have found a suggestion that a pre-existing state of induced resistance can be abrogated in a small number of cases by strain-specific parity. Some of the virgins and the females parous by BALB/c males from the previous experiment that failed to take the tumor were pooled and randomly divided into 3 groups as follows. One group was mated to DBA/2 males to produce from 2 to 6 litters, another to BALB/c males to produce from 1 to 5 litters, and the third was not mated. After a second challenge with the tumor, from 4 to 6 months after the first, the tumor took in 15 per cent (9/60) of the females mated to DBA/2 males, while none of the females parous by BALB/c males (0/24) and none of those not mated in this experiment (0/24) grew the tumor (9/60: 0/48; $P < 0.01$).

Regarding the characteristics and nature of this effect, we wondered whether the tolerance was specific for the genetic constitution of the male and whether it was influenced by the presence of the mammary-tumor-milk agent in the male. Accordingly, another experiment was performed, involving 5 groups of female recipients. One group was retained as virgins and each animal of the remaining 4 groups was mated to a male of one of the following strains or sublines: agent-bearing DBA/2, agent-free DBA/2_e, agent-bearing C3H, and agent-free BALB/c. After the parous females had had 1 or 2 litters they were immunized and challenged with the tumor. The tumor took only in those females parous by DBA/2 males, irrespective of whether the males did (15/42 = 36 per cent) or did not (17/37 = 46 per cent) carry the milk agent. The tumor did not take in virgins (0/40) or in females mated either to C3H (0/21) or to BALB/c (0/32) males (*see* TABLE 3).

TABLE 3

EFFECT OF PARITY FOLLOWING INTRA- AND INTERSTRAIN MATINGS BY AGENT-BEARING AND AGENT-FREE MALES UPON INDUCTION OF RESISTANCE TO TUMOR DBA49 IN BALB/C FEMALES*

Females previously mated to:	Milk agent in male	Takes/total	Takes (%)
DBA/2_e†	No	17/37	46
DBA/2†	Yes	15/42	36
C3H	Yes	0/21	0
BALB/c	No	0/32	0
Not mated	—	0/40	0

* Females were "immunized" with 0.05 ml. of DBA/2 blood 0 to 10 days after their last litter and challenged with the tumor 10 days later.

† Obtained from M. K. Deringer. "Agent-free" males were from a line that had originated from transferred ova.

Discussion

It has been reported that, in the case of normal tissues, skin homografts survive longer on pregnant rabbits[4] and mice.[5] Those studies, however, were not designed to disclose an effect produced as a consequence of the specific genetic constitution of the breeding male. In more detailed studies it was reported that homograft survival in pregnant or parous mice was not changed as a result of genetically specific matings.[6] In cattle, skin transplanted from calves to their dams often gave evidence of immunization of the dam as a result of pregnancy;[7] however, no such effect was found in rabbits or rats.[8]

Peer[9] has reported that the survival of skin grafts transplanted from child to mother was often greater than that of child-to-father grafts, which were rejected within an expected time interval. This finding is interesting, as it may indicate a persisting maternal desensitization or tolerance, and our results tend to support such observations. We found that both natural and induced resistance of female mice to transplantable tumors decreased only when parity was by males genetically related to the tumor. The ability to induce resistance in females parous by C3H males further indicates that the effect is strain-specific and is not merely the result of an interstrain mating. Earlier work on homologous tumors transplanted into parous or pregnant animals involved the use of randomly bred hosts or tumors of unknown origin, or both, and is therefore difficult to interpret in the present context.

Susceptibility to the tumor appeared to increase with multiparity by DBA/2 males; the data, however, were inconclusive. It has been suggested that the passage of fetal cells into the maternal circulation desensitizes the mother.[10] Presumably, this would have a cumulative effect through repeated pregnancies.

The effect of parity does not seem to be explained by hormonal changes incident to pregnancy, as it was found only in specifically mated postpartum females. The presence or absence of the milk agent in the male had no detectable influence. Preliminary studies indicate that the effect persists for at least 150 days after parturition and that it is not merely the result of exposure to the male in caging. The relationship of this effect to the tolerance phenomena reviewed by Owen[11] is uncertain, as our findings do not indicate the mechanism responsible for this change in adult females. It is interesting to note that acquired tolerance to skin homografts is transferred between isologous adult mice in parabiosis.[12,13]

These observations on the influence of parity can lead to much speculation—more than may be discussed in this brief paper. The phenomena of gestation and of homografting have a relationship in the use of terms, at least. Whether the similarities are more than a matter of words remains for further study. If the relationship is more than verbal, research may throw some light on problems of homografting, malignancy, and the complications of pregnancy.

Summary

Parity by DBA/2 males resulted in some degree of "tolerance" in postpartum BALB/c female mice to transplants of a DBA/2 tumor. Tolerance appeared to be not merely the result of an interstrain mating but to depend upon a genetic relationship between the sire of previous litters and the subsequent homologous graft. It was seen in "natural" resistance, but more strikingly in "induced" resistance. Tolerance seemed to increase with multiparity. In work in progress, the tolerance seems to be long-lasting and not induced by mere exposure to the male in caging, and it appears that a pre-existing induced immunity may be partially abrogated by strain-specific mating.

References

1. Billingham, R. E., L. Brent & P. B. Medawar. 1956. Quantitative studies on tissue transplantation immunity. III. Actively acquired tolerance. Phil. Trans. Roy. Soc. London. **B239:** 357–414.
2. Barrett, M. K., W. H. Hansen & B. F. Spilman. 1951. The nature of the antigen in induced resistance to tumors. Cancer Research. **11:** 930–936.
3. Barrett, M. K. & W. H. Hansen. 1957. Undulations in the time-response curve for tumor immunity after primary immunization with washed erythrocytes. J. Natl. Cancer Inst. **18:** 57–63.
4. Heslop, R. W., P. L. Krohn & E. M. Sparrow. 1954. The effect of pregnancy on the survival of skin homografts in rabbits. J. Endocrinol. **10:** 325–332.
5. Valone, J. A. 1952. The effect of gestation on experimental skin homografts. Plastic Reconstr. Surg. **10:** 354–364.
6. Medawar, P. B. & E. M. Sparrow. 1956. The effects of adrenocortical hormones, adrenocorticotrophic hormone and pregnancy on skin transplantation immunity in mice. J. Endocrinol. **14:** 240–256.
7. Billingham, R. E. & G. H. Lampkin. 1957. Further studies in tissue homotransplantation in cattle. J. Embryol. Exptl. Morphol. **5:** 351–367.
8. Woodruff, M. F. A. 1957. Transplantation immunity and the immunological problem of pregnancy. Proc. Roy. Soc. London. **B148:** 68–75.
9. Peer, L. A. 1958. Behavior of skin grafts exchanged between parents and offspring. Ann. N. Y. Acad. Sci. **73**(3): 584–588.
10. Billingham, R. E. & E. M. Sparrow. 1955. The effect of prior intravenous injections of dissociated epidermal cells and blood on the survival of skin homografts in rabbits. J. Embryol. Exptl. Morphol. **3:** 265–285.
11. Owen, R. D. 1957. Immunological tolerance. Federation Proc. **16:** 581–591.

12. Mariani, T., C. Martinez, J. M. Smith & R. A. Good. 1959. Induction of immunological tolerance to male skin isografts in female mice subsequent to neonatal period. Proc. Soc. Exptl. Biol. Med. **101:** 596–599.
13. Martinez, C., J. M. Smith, F. Shapiro & R. A. Good. 1959. Transfer of acquired immunological tolerance of skin homografts in mice joined in parabiosis. Proc. Soc. Exptl. Biol. Med. **102:** 413–417.

Discussion of the Paper

Rogers: Barrett's reference to Peer's work in humans with reciprocal skin homografting between parents and offspring and the unexplainable longer survival of offspring homografts on some parent recipients has also been verified by our laboratory in animals. We reported last year a corroboration of Peer's findings in our study of 474 rats that represented a variety of close genetic relationships. At that time we, too, found it difficult to explain a longer median survival time (MST) of some reciprocal skin homografts transplanted from daughters or sons to their mothers than the MST of maternal skin homografts placed upon these same offspring. There certainly must be some mechanism of fetal-maternal adjustment here that can be clarified only by those more intimately involved in a study of what happens to all the chromosomal and gene elements in cellular division when fertilization and subsequent embryonic development take place. Moreover, the concept of an exact quantitative 1:1 matching of histocompatibility genes between donor and recipient as a determinant of whether tissue transplants will survive or be rejected might also be subject to more questioning in such cases as these.

Barrett: Do you not think that something like this immune tolerance to the fetus must be going on? None of us doubts the laws of transplantation. The mother and the fetus must have some way of adjusting to one another in terms of this sort of reaction, and we have asked ourselves if this explains the discrepancy when a statistical geneticist tells us that about 16 per cent of matings *should* yield an erythroblastotic type of incompatibility in newborn children, but actually the true incidence is only 1.4 per cent.

Michael Galton (*Dept. of Pathology, Harvard University, Boston, Mass.*): I present some work that I think is quite closely related to Barrett's findings.

Immune tolerance to the fetus is a surprising feature, and thus far there has been no satisfactory explanation of it, although there have been many theories. I feel that by examination of the placenta itself, especially in humans, one may obtain an answer to this problem. The syncytial trophoblast, I think, may hold the secret, because this tissue is the only tissue of fetal genetic origin ever to come in contact with the maternal tissues.

We have interesting microscopic evidence supporting this theory. We recently inspected a section of the trophoblast of a placental villus obtained from a therapeutic abortion at fourteen weeks (the mother had suffered an attack of German measles). I was impressed by the fact that the outer layer of villus cells was much smaller than the inner layer of cells, the outer layer consisting of the *syncytial* trophoblastic cells, and the inner layer of the cyto-trophoblastic cells.

This particular microscopic section was stained by the Feulgen method, which stains DNA quantitatively. Microscopic examination of this section and other preliminary observations confirm the finding that the nuclei of the syncytial trophoblast contain only one half the amount of DNA found in the cytotrophoblast. In other words, there is good presumptive evidence that the syncytial trophoblast contains haploid nuclei—haploid in comparison to nuclei of the cytotrophoblast in telophase, which contain the diploid number of chromosomes.

The significance of this finding is important with general regard to the immunology of pregnancy. I therefore make two suggestions. First, one may believe in the "stem-cell" origin of the syncytial and cytotrophoblast, according to which the stem cell, at the genesis of this cell layer, undergoes a mitotic division in which there is a rejection of the paternal genetic element of chromosomes, only the maternal set being carried forward in these cells.

However—and here is where I think Barrett, Peer, and Rogers' observations may be pertinent—the alternative theory is that these nuclei or this cell layer is derived by some as-yet-unknown interaction between embryonic and maternal tissues, which may account for some of the cases of apparent immune tolerance described by these authors.

There is considerable evidence as to this finding, and the fact remains that these syncytial cells are smaller. They are in a syncytium for which there is no known explanation. They evoke no tissue reaction when they invade the endometrium, and they have no form of visible mitotic figures even though they are obviously in the very early stages of pregnancy when they will be multiplying with great rapidity.

PREHN: Barrett, in his usual thorough manner, has already largely given the discussion that I had planned to give. However, there are one or two comments that should perhaps be made. I think it quite apparent from the work of Medawar and Woodruff, which Barrett has cited, that a maternal tolerance is not essential for the maintenance of pregnancy. It also is quite apparent, I think, that Barrett obtained his extraordinary results by using a very particular biological system—a biological system that he has used to good advantage in the past and which perhaps possesses a number of peculiarities. One of these peculiarities is that the red cell is highly antigenic in this system, which may be important in explaining the results obtained.

The other is that the strains used are closely related genetically. The same H-2 locus is present; furthermore, Barrett is using a tumor as a test object, one which is very precariously balanced on the border line of growing or not growing, so that very minor influences, minor in the sense of magnitude but not importance, can be detected.

We ourselves were recently able to show that in this same system it was possible to produce some degree of tolerance by the injection of large doses of blood intravenously.

THE ANEMIA OF PARABIOTIC INTOXICATION*

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A mouse suffering from parabiotic intoxication has a wasted appearance; its fur is ruffled, its back is hunched, and it is strikingly anemic. This disease, when fatal, most commonly occurs when an F_1 hybrid mouse has been joined in parabiosis to a mouse of 1 parental strain, provided the parents differ at the H-2 locus. This is a genetic situation similar to that seen in homologous or "runt" disease. It is therefore not surprising that a number of investigators, including van Bekkum,[1] Trentin,[2] ourselves[3] and, most recently, Billingham,[4] have interpreted parabiotic intoxication as a phenomenon similar to runt disease. This has been expressed by Billingham in his lucid review in *Science* as follows: "As far as it goes, all the evidence suggests that there is a striking similarity between parabiosis intoxication and homologous or runt disease."[4]

If this striking similarity in external appearance and genetic background extends to its causation, parabiotic intoxication would result from an immune response of the pure against the hybrid. One would think in terms of antibodies produced either within the pure partner or by pure cells that have crossed into the hybrid and colonized its reticuloendothelial system. The anemia of intoxication could then be due to hemolysis of the hybrid's erythrocytes, with or without preceding agglutination; also, it could be due either to depression of the hybrid's bone marrow or to a combination of both.

We shall present data that rule out colonizing pure cells as the cause of the anemia, data that do not support circulating or cellular antibodies as being responsible. Furthermore, we shall demonstrate that the anemia is due neither to hemolysis nor to marrow depression. In short, our data cast doubt upon the concept that parabiotic intoxication is closely related to runt disease. Instead, we advance an alternate concept based on the data obtained by us. This concept is by no means new. Hall and Hall described it four years ago[5,6] as an alternate to the theories advanced by Chute and Sommers,[7] who observed incomplete isohemagglutinins.

It is a peculiar fact that parabiosis of mice from two unrelated strains usually does not terminate in fatal parabiotic intoxication, but results instead in the separation of the partners (FIGURE 1). Whenever partners differ at the H-2 locus, two seemingly independent reactions are set into motion: first, the closing down of vascular anastomoses, which endangers the texture of the junction and tends to result in separation of partners and, second, parabiotic intoxication. The first reaction interferes with the second, inasmuch as the closing down of anas-

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tomoses tends to diminish the intensity of the intoxication. In consequence, fatal intoxication is relatively uncommon when both partners attempt to close down anastomoses; this is the case with mice of most unrelated inbred strains. By contrast, when only one partner carries such an antigen, the closing down of anastomoses is somewhat delayed, and fatal intoxication of the hybrid is a most common result.

Materials and Methods

Animals. The experimental animals were C57BL/6, A, BALB/c, Street, CBA, and C3H-strain mice and their reciprocal F_1 hybrids obtained from our colonies, which have been maintained by brother-sister matings for many years.

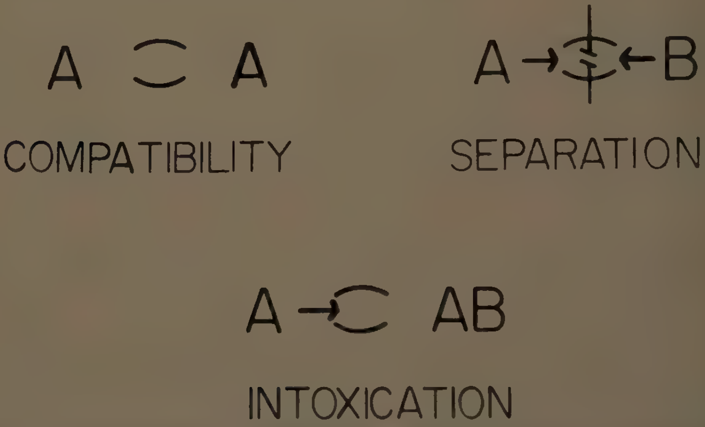

FIGURE 1. General patterns of parabiotic compatibility. *A* and *B* represent different mouse strains with different alleles at the H-2 locus.

Technique of parabiosis. This technique was essentially as previously described[3] except that skin edges were not joined by continuous absorbable suture but by staples dispensed from a commercial apparatus,* the lip of which was trimmed back in order to obtain freer action. This modification permitted joining many pairs of mice in a relatively short time and did not materially change the biological events observed after parabiosis.

Hematological data. We used hematocrit determinations instead of erythrocyte counts because of their greater ease and reproducibility. Blood samples were obtained from the tail vein, taken in heparinized capillaries, and spun down in a microhematocrit centrifuge. For reticulocyte counts an additional drop of blood was mixed with brilliant cresyl blue, incubated at room temperature for at least 20 min., and filmed out, and the number of reticulocytes per 1000 total red cells was counted. The animals were bled no more frequently than every fourth day, as bleeding at more frequent intervals was found to affect the hematocrit and reticulocyte levels both in normal and in parabiosed animals. This bleeding pattern explains certain

* Swingline "Tot 50," Swingline, Inc., Long Island City, N. Y.

irregularities of hematocrit levels seen in TABLE 1, since these values were obtained from different pairs that frequently differed as to the speed with which intoxication developed.

Analysis of hemoglobin. In order to determine the onset and degree of exchange of blood between partners, we used the method described by Popp *et al.*,[8] who analyzed mouse hemoglobins electrophoretically by the paper strip method, which revealed genetic differences. Specifically, the hemoglobin of mice of the C57BL strain showed a "sharp" pattern, while that of most other strains was "diffuse." The hemoglobin of an F_1 hybrid of a sharp and a diffuse strain showed both sharp and diffuse characteristics indistinguishable from an *in vitro* mixture of equal amounts of sharp and diffuse hemoglobins.

The mice were bled from the tail directly into a test tube containing 0.85 per cent saline and a drop of heparin. This was centrifuged and the supernate discarded. After 2 additional washings, the cells were hemolyzed by adding distilled water to give a volume equal to 10 times that of the packed cells.

Paper electrophoresis of the hemoglobin was carried out using the Beckman (Spinco) system of Durrum-type cell and Duostat-regulated power supply. A barbital buffer with a *p*H of 8.6 and ionic strength of 0.050 was used. A constant current of 5 ma was applied for 15 hours. The strips were stained with bromphenol blue dye.

Histological studies. When pairs were sacrificed, tissues were fixed in formalin and / or Zenker's fluid. Sections were stained with hematoxylin-eosin and, when warranted, with Prussian blue for iron-containing components.

Observations

Course of anemia. The data in TABLE 1 show a slight lowering of hematocrit values of both the pure and hybrid mouse the day after union, presumably due to surgical trauma. Starting on the fourth day and continuing until about the tenth day, the hybrid's hematocrit dropped precipitously; thereafter it became more or less stabilized. Simultaneously, the hematocrit values of the pure partner climbed. We reported this observation previously with respect to erythrocyte levels.[3]

Surgical separation of partners; transfer of blood and cell suspensions from pure partners. To obtain evidence either for or against the presence of immunologically active, colonizing, pure cells within the hybrids, pure-hybrid pairs were separated surgically at the time of intoxication, as determined by hematocrit levels of the hybrids. There was a rapid return toward normal levels between the tenth and the seventeenth days of union whenever the mice were separated (FIGURE 2). However, a few severely intoxicated hybrids died soon after separation, presumably because they were already severely debilitated.

Since this observation suggests that the intoxicating effect did not emanate from colonizing pure cells within the hybrid, it would then be necessary to relate it to structures within the pure animal proper. We exsanguinated pure mice, either while in parabiosis with intoxicated hybrids or after the death

TABLE 1
HEMATOCRIT AND RETICULOCYTE VALUES OF INBRED MICE AND THEIR F_1 HYBRID PARTNERS AFTER PARABIOTIC UNION*

Days after parabiosis		1	2	3	4	5	6	7	8	9	10	11	12	15	16	17
								Hematocrit								
Pure-strain partner	Mean	48	45	52	59	62	54	65	63	70	64	65	59	20	51	69
	Range	48	41–53	51–53	54–63	57–69	46–71	62–68	50–71	70	29–80	47–76	33–76	20	21–74	23–83
	No. sampled	2	7	2	2	4	9	2	3	1	12	8	9	1	3	9
F_1 hybrid partner	Mean	47	45	49	35	30	43	25	30	16	23	24	27	44	36	21
	Range	47	39–52	44–53	28–42	22–41	11–55	18–32	18–50	16	12–36	5–42	5–41	44	18–46	7–67
	No. sampled	2	8	2	2	4	10	3	3	1	9	7	9	1	3	7
								Reticulocytes								
Pure-strain partner	Mean	28	20	9	7	24	53	48	50	40	45	28	43	68	9	42
	Range	25–31	3–41	6–12	5–9	7–36	5–181	38–58	26–95	40	2–69	0–102	10–71	68	0–18	8–114
	No. sampled	2	8	2	2	4	9	2	3	1	10	9	9	1	2	8
F_1 hybrid partner	Mean	30	11	23	10	35	40	117	230	265	99	297	379	12	454	544
	Range	23–36	1–25	19–26	8–12	28–44	2–126	48–244	33–368	265	3–371	0–675	0–872	12	18–890	19–843
	No. sampled	2	8	2	2	4	9	3	3	1	7	6	8	1	2	7

* Results were obtained from several pilot experiments in order to avoid the daily bleeding of the same pairs.

of the intoxicated partner, and injected the blood into stock hybrid mice of like genetic background. Neither single nor multiple injections affected the hematocrit levels of the hybrid mice. We were as unsuccessful when

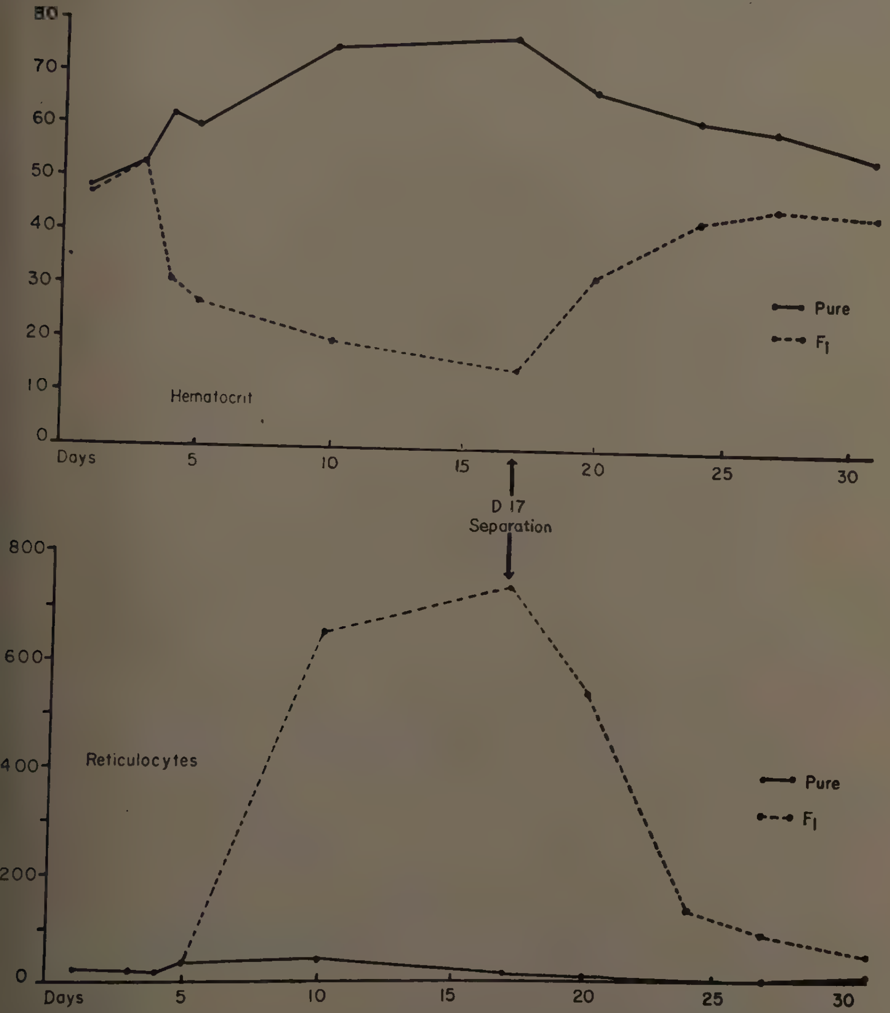

FIGURE 2. Hematocrit and reticulocyte values of 4 parabiotic pairs of BALB/c and BALB/c × C57BL)F_1 mice, showing the effect of surgical separation on the seventeenth day f union.

ising suspensions of spleen or lymph nodes from the intoxicating mice, whether ested within hours, days, or weeks after transfer.

Bone marrow depression versus blood loss. To determine whether the anemia vas caused by blood loss or depression of the marrow, we compared the hematocrit and reticulocyte values of the anemic hybrid with these values n a group of mice exposed to sublethal irradiation (500 r) and in another group of mice that had suffered severe blood loss through multiple severe bleedings (FIGURE 3). The drop in hematocrit values of the hybrid during the crucial period between the fifth and tenth days was steep and resembled that of the exsanguinated mouse more closely than that of the irradiated mouse, which showed a less steep decline. The similarity between the intoxicated

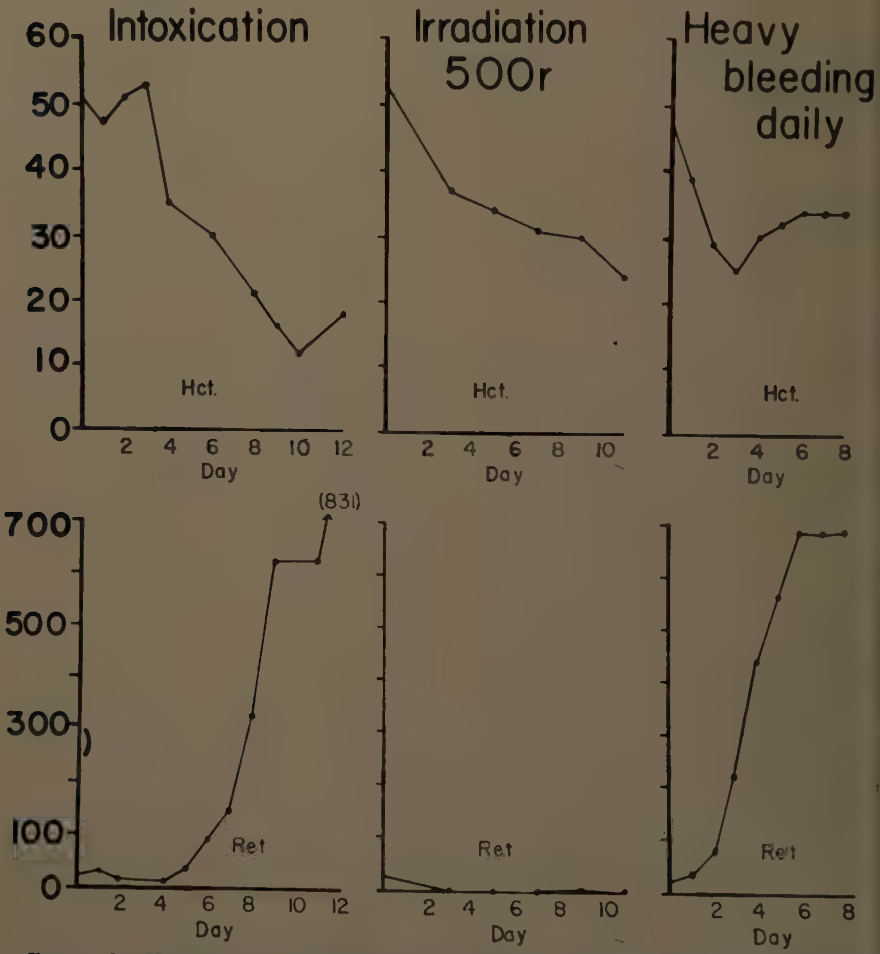

FIGURE 3. Hematocrit and reticulocyte values of groups of mice in parabiotic intoxication, following sublethal irradiation, and after heavy daily bleedings. The values in parabiotic intoxication resemble those following heavy daily bleedings.

and the exsanguinated mouse was much more striking when the reticulocyte values were compared. Both showed a massive and persistent wave of reticulocytes, reaching levels up to 90 per cent and indicating an active and highly responsive erythroid apparatus. Reticulocyte values in irradiated mice dropped to zero and reappeared slowly, beginning on the ninth day after irradiation. Further evidence against the presence of depressed bone marrow activity was obtained from histological study of the marrow of intoxicated hybrids, which revealed a highly cellular, active organ.

Since there was no evidence of external blood loss, the anemia could have been caused by massive hemolysis. In order to determine the symptoms caused by massive hemolysis, daily doses of phenylhydrazine (0.04 to 0.08 mg./gm. body weight) were injected intraperitoneally. A rapid drop in hematocrit occurred, although it did not reach the levels seen in intoxicated hybrids. In addition to a distinct pallor, the symptoms noted were a faint jaundice of the paws, ears, and subcutaneous tissue, deep-brown discoloration of the urine, and deep-yellow serum. The spleens were markedly enlarged, frequently exceeding 1 gm. (FIGURE 4). The external and cut surfaces of the spleen were almost black, while the liver was a pale grayish brown. Histological sections showed numerous macrophages containing golden-brown,

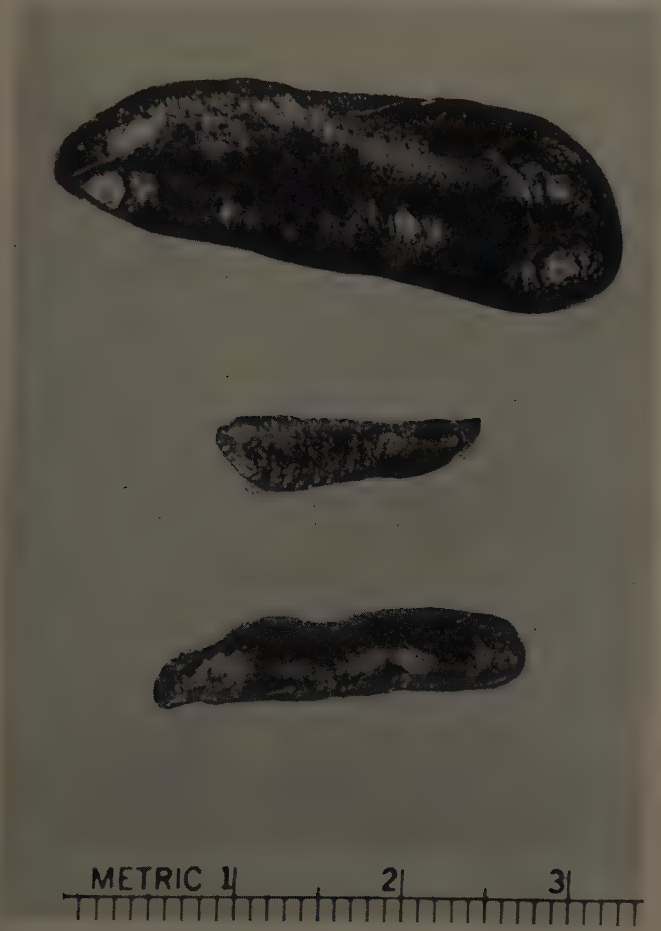

FIGURE 4. (*Bottom*) Spleen of mouse in parabiotic intoxication; (*center*) spleen of normal mouse; (*top*) spleen of mouse treated with phenylhydrazine.

iron-containing pigment in the red pulp of the spleen; the Kupffer's cells of the liver were hypertrophied and filled with similar pigment. Neither the urine nor the serum of the intoxicated hybrids (or their pure partners) showed comparable changes. Their spleens, although moderately enlarged, revealed only minimal amounts of iron-containing pigment, similar to the amount seen in normal spleens. These observations do not support massive hemolysis as the cause of the anemia.

The absence of hemolysis means either that the hybrid's erythrocytes did not pass into the pure partner's circulation or that they were unharmed in surroundings in which they should have been hemolyzed. The answer was readily obtained by electrophoretic typing of the hemoglobin of parabiosed mice with sharp and diffuse hemoglobin (C57BL and Street), or with sharp and sharp-diffuse hemoglobin (C57BL and [C57BL × Street] F_1). Typing showed the presence of foreign hemoglobin on the third day of union in some pairs and persistently thereafter in all pairs. In most instances there appeared to be equal amounts of foreign and native hemoglobin from the fourth day on, which was suggestive of complete mixing of blood. Beginning in the second week there was a gradual return to the native state, whether or not the union of the partners persisted during this period. The reversal was complete by the sixteenth day in one instance and by the thirtieth day in most animals. There was no evidence of a sudden disappearance of foreign hemoglobin suggestive of a hemolytic episode; instead, red cells appeared to live their normal life span. This agrees with Owen's earlier observation of parabiosed rats, studied by means of the technique of differential agglutination.[9]

Polycythemia of the pure partner. Insufficient attention has been paid to the polycythemia of the pure partner. We considered it likely that its cause was the spilling over of erythropoietic principle from the anemic hybrid into the pure partner. However, we were unable to elicit polycythemia in stock mice injected with cell suspensions from spleen and lymph nodes of intoxicated mice. Also, there was no time interval between the hybrid's anemia and the pure animal's polycythemia that would have been expected if erythropoietic principle were elaborated in response to blood loss. The complementary relationship between the anemia and polycythemia was most apparent when individual pairs were compared. In the course of intoxication, when there were episodes of recovery of the hematocrit level in the hybrid, this recovery was always accompanied by a decrease of the hematocrit level in the pure partner. Likewise, in occasional "paradoxical" pairs, an increase in the hybrid's hematocrit was associated with a decrease in that of the pure partner. In essence, the combined red cell mass per volume remained constant if one allowed for a small initial blood loss subsequent to the surgical procedure (FIGURES 5 and 6). These data suggest a shift of the red cell mass from the hybrid to its pure partner at about the fifth day of union.

The shift. The possibility of a shift as a cause of the hybrid's anemia was mentioned by Chute and Sommers[7] and also by Hall and Hall;[6] the former authors theorized that the sludged antibody-coated erythrocytes of the hybrid are mechanically trapped within its pure partner and are unable to "re-negotiate the small vascular connections between the parabionts. . . ." This theory is at variance with our electrophoretic data. However, the thought of a shift per se is in line with all our data and seems inescapable.

How can such a shift come about? We submit that a primary shocklike dilatation of the vascular bed of the pure partner could explain a good many, and possibly all, of the symptoms of parabiotic intoxication. The dilatation would create a pressure gradient followed by an inflow of blood from the ybrid to the pure. The hybrid would attempt to compensate for the blood oss by shifting tissue fluid into its circulation, resulting in lowered hematorit values. The hybrid's bone marrow would respond by overproduction f reticulocytes, some of which would spill over into its pure partner. This ypothetical process of vasodilatation and hybrid-to-pure flow would continue t an increased rate until the first half of the second week, when anastomoses egin to close down. A leakage sufficient to maintain the imbalance still ccurs thereafter, but as a rule the imbalance does not increase. The hyrid's death usually occurs from loss of blood or intercurrent disease, unless he immune response comes to its rescue and seals off all avenues of com-

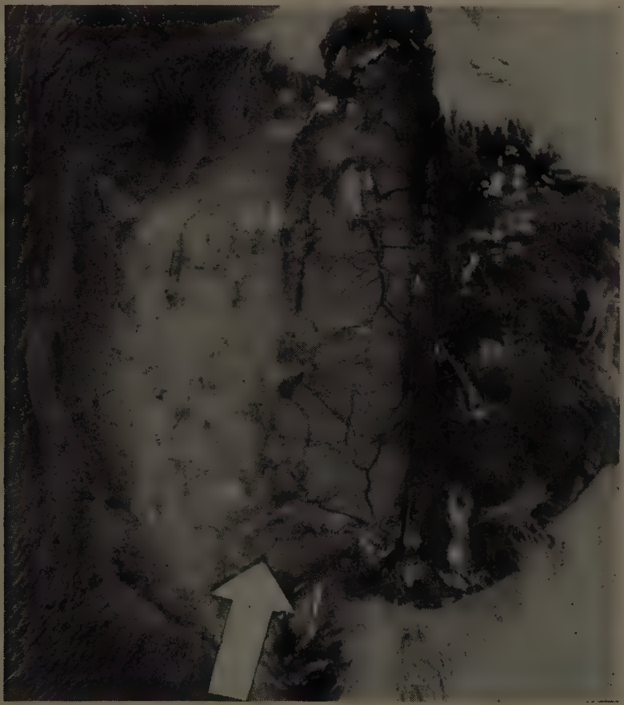

FIGURE 5. Line of junction (*arrow*) of pure-F_1 hybrid pair at the height of intoxication The skin of the pure partner (white) has been reflected to show engorgement of subcutaneous issue. There is marked pallor of the subcutaneous tissue of the hybrid (pigmented) partner.

munication. One might be tempted to say that parabiotic intoxication, in contrast to runt disease, comes about not because of the immune response but in spite of it, although it is, of course, triggered by an immune response and does not occur in the absence of an appropriate genetic diversity. As yet we have not proved the occurrence of the hypothetical vasodilatation.

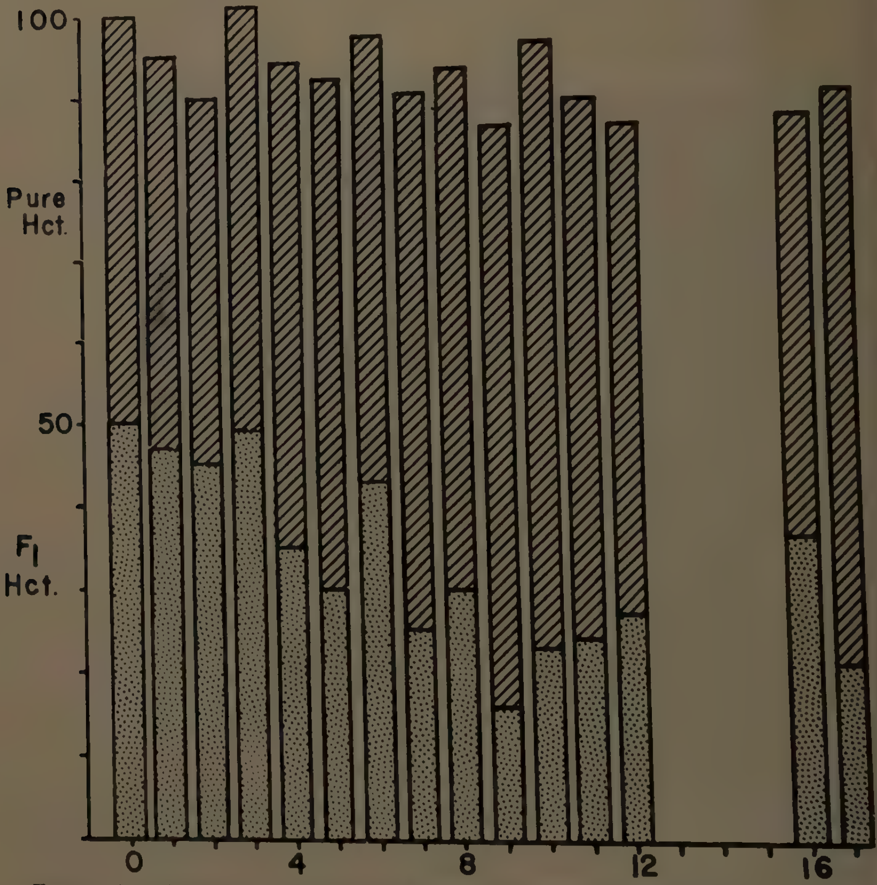

FIGURE 6. Histogram of the combined hematocrit values of pure-F_1 hybrid pairs. The combined values remain relatively constant throughout the course of intoxication.

However, Hall and Hall produced the picture of parabiotic intoxication in littermate rats by transection of the spinal cord of 1 partner.[6]

We also draw attention to a phenomenon that we called early disease for the want of a better term. Early disease is early death of the pure partner of a pure-hybrid parabiotic pair at the end of the first week, particularly when the pure partner is a BALB/c strain mouse. At death, these mice frequently reveal a dilated heart and, in one instance, thrombosis of a cardiac atrium, consistent with overloading of the circulation and heart failure.

Summary

We doubt that parabiotic intoxication is the direct effect of an immune response against an incompatible partner and thereby comparable to runt disease.

An intoxicated mouse shows severe anemia and a marked reticulocyte response, while the partner has a severe polycythemia. Surgical separation of the intoxicated hybrid from its pure partner results in a rapid change toward normal blood values. This observation does not suggest colonization of the intoxicated mouse by the cells of its partner. Transferring blood, spleen, or lymph node cells from an intoxicating mouse into stock animals does not cause anemia in them. Consequently, an immune response is not likely to be the cause of the anemia.

The anemia is not due to bone marrow depression, but is apparently the result of blood loss. However, gross and microscopic study of the partners and electrophoretic typing of the hemoglobin do not support hemolysis as the cause.

The data obtained suggest that the symptoms of intoxication are due to a massive shift of red cell mass from the intoxicated mouse to its partner. The term parabiotic intoxication therefore does not seem appropriate.

Acknowledgments

We are indebted to Wanda Reynolds, Michael Strainer, and Carol Brain, Junior Fellows, Montana Division, American Cancer Society, for technical assistance.

References

1. Van Bekkum, D. W., O. Vos & W. W. H. Weyzen. 1959. The pathogenesis of the secondary disease after foreign bone marrow transplantation in X-irradiated mice. J. Natl. Cancer Inst. **23:** 75.
2. Trentin, J. J. 1959. Homologous disease in unirradiated F_1 hybrid mice receiving parental lymphoid tissue. : 207. *In* Biological Problems of Grafting. Univ. of Liége. Liége, Belgium.
3. Eichwald, E. J., E. C. Lustgraaf & M. Strainer. 1959. Genetic factors in parabiosis. J. Natl. Cancer Inst. **23:** 1193.
4. Billingham, R. E. 1959. Reactions of grafts against their hosts. Science. **130:** 947.
5. Hall, C. E. & O. Hall. 1956. On the nature of parabiosis intoxication: shock as the precipitating cause. J. Exptl. Med. **103:** 263.
6. Hall, C. E. & O. Hall. 1957. Postseparation recovery from parabiosis intoxication in the rat. Am. J. Physiol. **189:** 495.
7. Chute, R. N. & S. C. Sommers. 1952. Hemolytic disease and polycythemia in parabiosis intoxication. Blood. **7:** 1005.
8. Popp, A., G. E. Cosgrove, Jr. & R. D. Owen. 1958. Genetic differences in hemoglobin as markers for bone marrow transplantation in mice. Proc. Soc. Exptl. Biol. Med. **99:** 692.
9. Owen, R. D. 1956. Erythrocyte antigens and tolerance phenomena. Proc. Roy. Soc. London. **B146:** 8.

Discussion of the Paper

Silvers: I must say that I thoroughly enjoyed the presentation by Eichwald. You have attempted to find out whether any of the lymphoid cells from the parental strain are introduced into the adult hybrid by looking for an anemia that might result from such a passage. Is that right?

EICHWALD: They are not anemic.

SILVERS: Would it not be a more sensitive test if one introduced lymph-node cells from the F_1 hybrids, which may be of two genotypes if parental cells have obtained transfer, into newborn animals and looked for runt disease. I think it is possible that this would constitute a more sensitive test than the one you have done.

Another question I ask is suggested by the beautiful work of Boyce and Gorer, who have shown that if immunologically competent Strain *A* cells from animals immunized against C57 into C57/A hybrids are introduced, these cells are able to kill the F_1 hybrid, but if immune C57 cells are introduced into the F_1 hybrid, the *cells* seem to die. I wonder whether the C57 Black have been used in any of your combinations and, if so, do the results agree or disagree with these findings of Boyce and Gorer?

EICHWALD: Most of ours were C-57 Black, but we did not have too many AB's, did we? No. That would be interesting to know.

BILLINGHAM: In studying what are essentially homograft reactions "the wrong way round," such as runt disease, homologous disease in irradiation chimeras, and possibly parabiosis intoxication, which we all agree are of immunological origin, it must be pointed out that although we can put in a nice histologically and quantitatively definable reacting system, such as a cellular homograft of thoracic duct cells or bone marrow cells, the whole animal presents an exceedingly complex "antigen." Certainly, it lends itself to careful pathological elucidation of many obvious abnormalities, but we must be exceedingly cautious in deciding which are of primary and which of secondary importance. What damage is of direct immunological origin and what may be due to nutritional or physiological derangement? We still cannot explain the exact mechanism of destruction of a skin homograft, which is a simple system for pathological studies at the cellular level. When we make the entire host the target of the immunological reaction of inoculated immunologically competent cells, how much more difficult becomes the problem of interpreting the disease they cause!

B. A. RUBIN (*Baylor University, Houston, Texas*): We have taken the surviving animals in cases where a parabiosis failed, that is, where the hybrid died of a wasting type of disease, and we have found that in these cases the parent survives well, without too much damage. After a short time, we parabiosed them again: that is, we re-parabiosed the survivors to similar hybrids and, in some cases, we tried skin homografting them with hybrid skin.

I am sure that no one will be surprised at the results. In these cases, the second parabiosis was very rapidly rejected as were the skin homografts, in what I presume is a very typical second-set immunological reaction.

I am interested in these contradictory results, however, describing animals seemingly poised on a sort of knife edge, on which they cannot remain in a neutral immunological position, but must react strongly in one direction or the other.

I believe that Eichwald's observations may be part of the answer but, in any case, I think this "either-or" balance seems definitely to be affected by the method of parabiosis employed. The crucial matter here is not that there is a failure of eventual blood flow; what matters here is the rapidity of the exchange of antigenic material. With the coelomic type of anastomosis there is probably a much more rapid initial exchange of antigens.

William Damashek (*Department of Medicine, Tufts University School of Medicine, Boston, Mass.*): I am very happy to hear that Eichwald has taken up Hall's original experiment, in which we have been very much interested, as this situation of a severe anemia in one animal and marked polycythemia and high red cell count in another, is seen in some conditions of identical twinning, where it leads to death very frequently at birth or prior to birth. In such twins, this situation is generally due to a common arteriovenous fistula. I have wondered whether you might have investigated qualitatively the nature of these anastomoses to see if there is any such arteriovenous fistula in the parabioses you have studied.

Comment: Foı parabiotic pairs we have used AKRs and C3Hs. We have now parabiosed approximately 60 pairs, and our findings have been quite uniform. The AKR mouse is the one mouse in the pair that dies from about 10 days after parabiosis to as long a period as 56 days after the operation. The AKR mouse becomes grossly anemic, with hemoglobin in the range of 3 gm. per cent appearing about 7 days after the operation, and it remains in this condition until death. Interestingly, in the very long surviving pairs, the C3H mouse becomes anemic, as does the AKR mouse.

This raises an interesting point in view of the finding that the AKR mouse is always anemic. The C3H mouse is not always polycythemic; therefore, I wonder whether Eichwald's explanation is the only possible one to be considered here. There is, of course, another possibility—that blood may be lost. If animals become polycythemic, it is known (in the rat, for instance) that they may lose blood from the gastrointestinal tract. We have not yet looked for this, but I think it might be worth investigating. It may explain why the C3H mouse ultimately becomes anemic, as does the AKR mouse.

We have also checked on the white counts in these animals, and we find two interesting things. First, we get a leukopenia in the AKR mouse as well as its anemia, and this leukopenia may be an extremely severe one with white counts as low as 500 cells/cm.

On the other hand, the C3H mouse does not develop any markedly increased number of white cells and usually has a normal count, a lower count, or a slightly elevated count.

I mention these findings because I think they may be relevant, and I believe that we cannot solve this problem too easily.

Here is a question I put to Eichwald: Why does one mouse, the hybrid, develop the vasodilatation, and can we be sure that this is a true vasodilatation? Blood-volume studies should be important in these cases; what we may be observing is the appearance of a polycythemic animal. When our C3H animals have been polycythemic, they have shown the same appearance as Eichwald's animals. One then can observe the marked difference between the dead white skin—when you separate the animals—of the AKR mouse, on the one hand, and the extraordinary plethoric vascular lining of the skin in the C3H mouse. I wonder whether this isn't just an expression of polycythemia.

EICHWALD: Most of these questions have troubled me also. We have analyzed the feces of both partners quantitatively for fibrilinogen, and we did not see anything that suggested a loss of blood by one specific route or another. I wish I knew the answers to this problem.

IMMUNOLOGICAL EFFECTS OF MULTIPLE EXPERIMENTAL EMBRYONAL PARABIOSIS IN BIRDS*

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The observations of Owen[14] on the immunological characteristics of dizygotic bovine twins and the demonstration that the phenomenon was due to vascular placental anastomosis opened a most fruitful field of investigation in transplantation immunity. Since this is an example of spontaneous embryonal parabiosis and since the studies of Billingham *et al.*[1] and Simonsen[15] on induced tolerance in embryos and newborns have indicated different requirements for its induction in laboratory animals, the production of experimental embryonal parabiosis for this purpose is a worthwhile endeavor.[5,13]

It is known that birds produce large amounts of antibodies after the injection of foreign cells and, since they present the advantage of being independent during embryonal life from the mother's organism, they are ideally suited for studies of this type.

The original investigations of Hašek,[6,7] uniting in parabiosis pairs of chick embryos or chick with turkey, duck, or pheasant embryos, confirmed that various degrees of tolerance could be produced in birds by blocking the production of specific antibodies in embryos and that this acquired tolerance would persist in the adult animals. Hašek's demonstration of their inability to form immune agglutinins to the erythrocytes of their parabiont and the dissociation in birds between cellular chimerism and tolerance are especially interesting in view of the recent reports on "runting" of Simonsen,[17] Billingham and Silvers[2] and Siskind and Thomas[18] since, as both embryos are immunologically immature, graft-against-host reaction is not likely to occur.

The purpose of this paper is to report investigations in the creation of multiple bird parabionts by synchorial vascular anastomosis in different combinations. This was done in order to expose each member of the parabiotic team to a number of different antigens and to study the degree of the resultant tolerance by serologic methods and from the survival time of full-thickness skin grafts.

Method

Fertilized White Leghorn and Red Rhode Island eggs obtained from commercial breeders were incubated at 98° F. under standard conditions. On the fifth day of incubation they were candled and those without well-developed air chambers were discarded. On the tenth day the positions of the largest chorioallantoid vessels were determined by transillumination and indicated on the shell surface with a pencil mark. The eggs were carried to a tissue-culture room and their shells were sterilized by immersion in aqueous 1 per cent solution of Zephiran chloride,† rinsed twice in sterile distilled

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* The studies reported in this paper, done during the tenure of an Established Investigatorship of the American Heart Association, New York, N. Y., were supported by a grant from that association.

† Winthrop Laboratories, New York, N. Y.

water, and flamed after an application of absolute ethyl alcohol. A 4-by-7-mm. hole was then made at the indicated point by means of a portable electric saw with a sterilized blade. The shell membranes were removed with fine sterile forceps after a 1-mm. hole had been drilled over the air chamber to assure a better separation of the chorioallantoid membrane from the shell, and 1 ml. of sterile Ringer's solution was put in the hole. When a sufficient number of eggs was so prepared, the embryos were united by a portion of the blastoderm of a 48-hour-old chick embryo, attention being paid to its contact with the chorioallantoid membranes of both embryos to facilitate the formation of vascular anastomosis. The shells were joined with a mixture of 3 parts of beeswax and 1 part of paraffin, and the hole drilled over the air chamber was closed with a drop of the same material.

Multiple parabionts were united thus in teams of 3 to 10 eggs of the same or different breed and returned to the incubator (FIGURE 1). Alternatively to facilitate a closer antigen exchange between different members of a team, they also were united in a circular form (FIGURE 2).

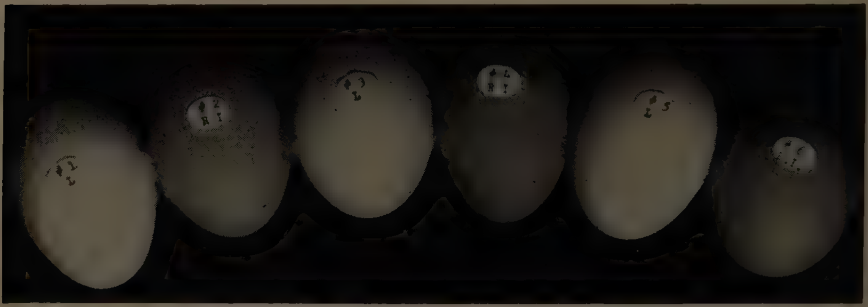

FIGURE 1.

Finally, a single embryo was joined to 3 partners that were not connected to each other, for the purpose of increasing the amount of antigen exchange by a single parabiont (FIGURE 3).

In all cases care was taken to prevent an undermining of the strength of the shell and to avoid covering too much of its surface with the wax preparation.

At different stages of incubation the presence of synchorial vascular anastomosis was demonstrated by injection into one of the allantoid veins of one of the partners of sterile solutions of Evans' blue or fluorescein.

At hatching, the chicks were marked with plastic pigeon rings, maintained in a brooder for two weeks, and then transferred to ordinary metal cages and given Purina chick Startena ad libitum. Agglutination tests were made on depression slides at different intervals to determine the presence or absence of antibody production, by the formation of isoagglutinins after immunization with erythrocytes of parabiotic partners or unrelated chicks. To demonstrate differences in the antigenic composition of the erythrocytes of members of a team, control chicks were used for the absorption of isoimmune serum prepared in a control bird.

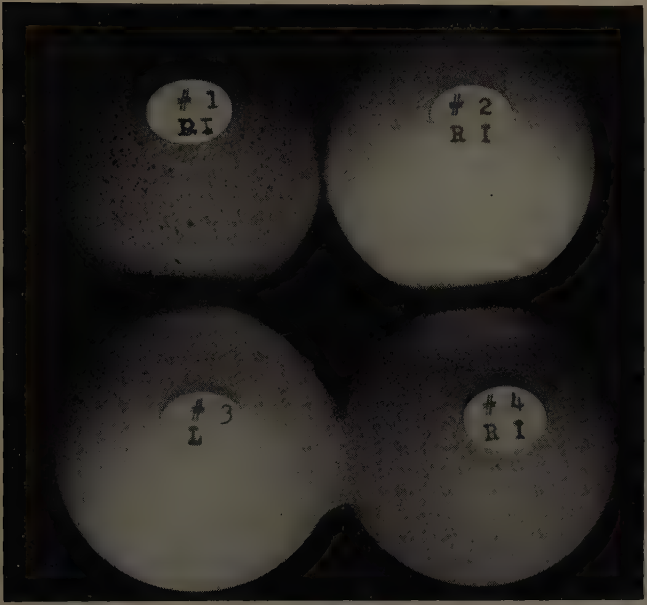

FIGURE 2.

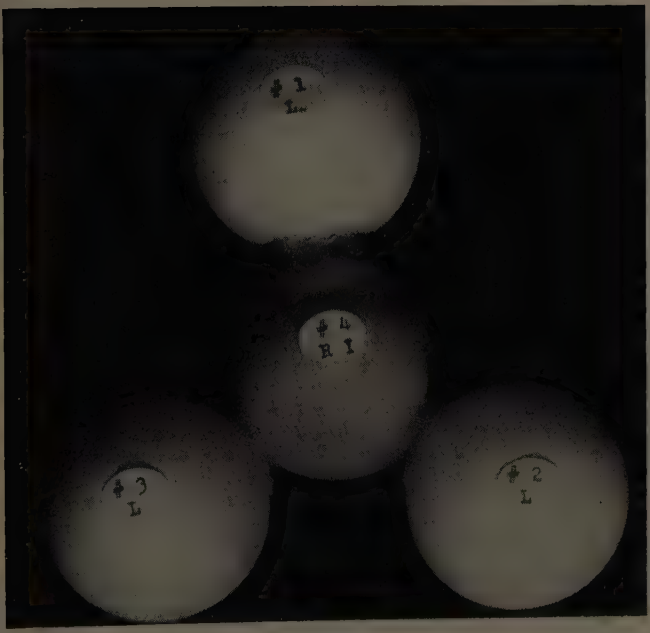

FIGURE 3.

Twenty-eight days after hatching, the chicks were anesthetized with ether and the down of their backs plucked. The skin was prepared with a 1 per cent alcohol solution of iodine after washing with pHisoHex.* Under sterile conditions three 10-by-10-mm. full-thickness skin squares were removed by careful dissection from the underlying tissue to avoid subcutaneous bleeding, 1-cm.-wide bridges of normal skin being left between them. The specimens were soaked in Ringer's solution and tattooed with India ink. The cephalad graft was rotated 180° and implanted as a control autograft on the caudal defect with interrupted 5-0 silk sutures. The cephalad defect was repaired with a caudad graft taken from a parabiotic partner prepared in similar manner, and the central defect received a graft from an unrelated donor of the same age. Each bird therefore had 3 full-thickness skin grafts. These were covered with collodion and nonpick ointment to prevent cannibalism. The chicks were marked with numbered aluminum bands in their wings and returned to their cages. The grafts were checked periodically and the growth of feathers or any gross morphologic changes recorded. Skin biopsies were made once a month or when evidence of graft rejection appeared. The specimens were processed by standard histological methods and evaluated in accordance with their morphologic characteristics, including elastic fiber pattern and cellular preservation. All animals were observed for 100 days or more for tabulation of skin survival rates.

To study the fate of full-thickness skin homografts in control chicks not united by synchorial parabiosis, a group of White Leghorn and Red Rhode Island chicks from the same supplier was treated, 4 weeks after hatching, with rotated autografts and unrelated homografts by the technique described above.

Results

The over-all survival rate of 182 chick embryos connected in multiple parabiosis was 72 per cent when joined on the tenth day of incubation.

The highest mortality occurred with the type of connection illustrated in FIGURE 3 (34 per cent). When Red Rhode Island or White Leghorn embryos were joined to others of the same breed, the proportion of eggs that hatched was greater than that in interbreed combinations.[12]

The presence of vascular anastomosis between embryos was demonstrated by vital stains injected into one of the mates, and their concentration in the different members of a team was found to be directly proportional to their distance from each other. When members were separated by 6 or more parabionts, no exchange of fluorescein or Evans' blue was observed.

These anastomoses also could be demonstrated in the emptied shells after hatching (FIGURE 4), the chorioallantoid vessels of each partner forming an intricate vascular network at the site of interembryonal connection.

Attempts to demonstrate the formation of isoagglutinins in adult parabionts injected with erythrocytes of a teammate failed.

Although in a 3-chick combination the parabionts still formed isoagglutinins when immunized with erythrocytes from a nonparabiotic homologous donor, 4 or more parabionts on a team drastically diminished formation of isoagglutinins to the erythrocytes of control chicks in any of the partners.

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* Winthrop Laboratories, New York, N. Y.

Differences in antigenic composition of erythrocytes in these chicks still could be shown by the absorption of immune serum in ducks.

When these animals were tested with full-thickness skin grafts, the survival rates of side-to-side parabionts were almost as high as those of control autografts (TABLE 1). The distance between parabionts during embryonal life slightly diminished the number of permanent takes.

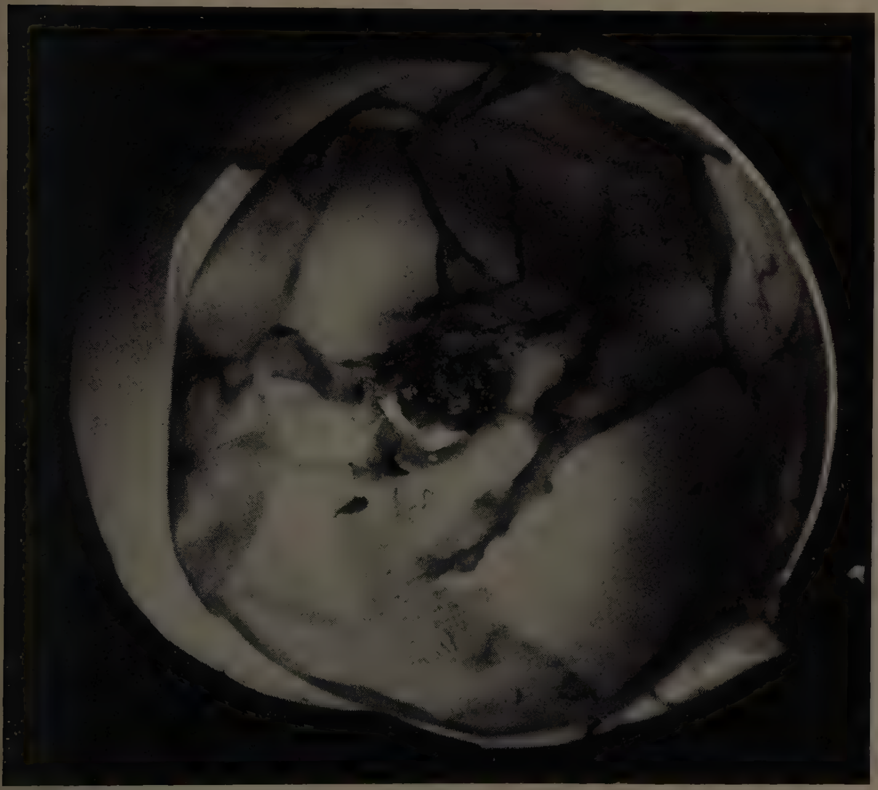

FIGURE 4.

The 100-plus days' survival rates of grafts from nonparabiotic homologous mates to recipients of a multiple team (4 or more) were approximately similar to those of "long-distance" parabionts.

No greater survival rate among nonparabiotic heterografts (duck to chick) was observed in these animals.

These results were in sharp contrast with the standard survival time of control skin homografts in nonparabiotic birds obtained from the same breeder (TABLE 2), in which no homograft survived for more than 22 days.

The variations in survival rate of grafts in heterosexual combinations were not significant.

Discussion

Since the results obtained by Cannon and Longmire[4] have indicated that a prolonged survival of full-thickness skin homografts in noninbred chicks could be achieved by grafting within the first 2 weeks of life, only 4-week-old chicks were subjected to transplantation to ensure that nonspecific tolerance would not take place independently of the embryonal exposure to a foreign antigen. Our results in control chicks indicate that such a period is adequate to avoid false positives.

TABLE 1

PER CENT DISTRIBUTION OF SURVIVAL TIMES IN DAYS OF FULL-THICKNESS SKIN GRAFTS IMPLANTED IN 4-WEEK-OLD CHICKS

Donor/recipient relationship	Distribution (%)*				
	<15	>15 ≦20	>20 ≦40	>40 ≦100	>100
Multiple parabiosis					
Side-to-side parabiosis	—	—	1	2	97
Separated by 1 parabiont	—	—	3	4	93
Separated by 2 parabionts	—	1	2	8	89
Separated by 4 parabionts	—	—	4	7	89
Separated by 6 parabionts	—	2	6	4	88
Nonparabiotic homologous donor	—	3	7	4	86
Nonparabiotic heterologous donor (duck)	92	7	1	—	—
Control autograft	—	—	—	2	98

* Mean ± standard error of the mean.

TABLE 2

FULL-THICKNESS SKIN HOMOGRAFTS ON 28-DAY-OLD CONTROL CHICKS

Breed	No. birds	Distribution* of survival times (days)			
		<10	>10 ≦15	>15 ≦20	>20
White Leghorn	32	18	9	4	1
Red Rhode Island	30	21	6	3	—

* Mean ± standard error of the mean.

† Ninety-six per cent less 100-day survival of control autografts.

Due to recognized difficulties in the evaluation of skin graft survival, 100 days, suggested by Billingham and Silvers,[3] was considered an adequate period for evaluation, although it was understood that long-term rejections still could occur.

As in Hašek's work with single parabiotic pairs,[6-8] no antibody production could be induced by reciprocal immunization of adult ex-mates in multiple parabiosis. Such parabionts, however, still produced high agglutinin titers when immunized with interstrain erythrocytes.

Hašek *et al.*,[9,10] in their studies of the production of tolerance by interspecific embryonal parabiosis, have shown that heterologous tolerance, the capacity for specific recognition of foreign antigens, is not maintained and that a tolerant animal does not recognize individual differences within its own species, thus establishing a fundamental difference between the specificity of homo- and heterotransplantation tolerance.

Our results with embryonal multiple parabiosis seem to indicate that, when a sufficient number of immunologically immature cells from different donors is put into contact, a nonspecific tolerance may be established, allowing permanent survival of grafts from random donors of the same species.

This method has the advantage that the graft-against-host reaction cannot occur as it does in transplantation tolerance induced with mature cells since, in fact, a mutual induction of tolerance takes place.

This is in agreement with the finding of Hašek *et al.*[10] and of Haškova *et al.*[11] that immature lymphoid cells injected into newborn animals will not produce runt disease and with the observations of Simonsen[15-17] and Billingham and Silvers[2] on the effects of adult cells in the embryo.

If these results can be extended to higher animals, the induction of nonspecific tolerance by polyantigenic immature cells or cell components in suitable recipients may provide investigators with valuable information on host-against-graft and graft-against-host dilemma.

Summary

Multiple experimental parabiosis in chick embryos was effected by a modification of the method proposed by Hašek for single parabiotic pairs. The presence of blood exchange between the embryos was demonstrated during and after hatching. The amount of antibody production by reciprocal immunization in birds joined during embryonic life was proportional to their spatial relationship during incubation. When a single embryo was united to three or more parabionts, successful exchange of full-thickness skin grafts could be carried out, not only among members of the parabiotic teams, but also among unrelated homologous donors. Survival times of heterologous skin grafts were similar to those observed in control animals.

References

1. Billingham, R. E., G. H. Lampkin, P. B. Medawar & H. Williams. 1952. Heredity. **6:** 201.
2. Billingham, R. E. & W. K. Silvers. 1959. J. Immunol. **82:** 448.
3. Billingham, R. E. & W. K. Silvers. 1959. Transplantation Bull. **6:** 399.
4. Cannon, J. A. & W. P. Longmire. 1952. Ann. Surg. **135:** 60.
5. Danforth, C. H. & F. Foster. 1929. J. Exptl. Zool. **52:** 443.
6. Hašek, M. 1953. Českoslov. biol. **2:** 267.
7. Hašek, M. 1957. Transplantation Bull. **4:** 113.
8. Hašek, M. & V. Haškova. 1958. Českoslov. biol. **7:** 282.
9. Hašek, M., T. Hraba & J. Hort. 1958. Ann. N. Y. Acad. Sci. **73**(3): 570.
10. Hašek, M., T. Hraba & J. Hort. 1959. Nature. **183:** 1199.
11. Haškova, V., I. Hilgert, M. Hrubesova & Z. Pokorna. 1959. Folia Biol. (Prague) **5.**
12. Lazzarini, A. A. 1958. Ann. N. Y. Acad. Sci. **73**(3): 575.
13. Lillie, F. R. 1916. Science. **43:** 611.
14. Owen, R. D. 1945. Science. **102:** 400.
15. Simonsen, M. 1953. Thesis. Munksgaard. Copenhagen, Denmark.
16. Simonsen, M. 1955. Nature. **175:** 763.
17. Simonsen, M. 1957. Acta Pathol. Microbiol. Scand. **40:** 480.
18. Siskind, G. W. & L. Thomas. 1959. Bull. Soc. Intern. Chir. **18:** 208.

Part II. Variations in the Response to Tissue Transplants

EFFECT OF SURGICAL ABLATION OF REGIONAL LYMPH NODES ON SURVIVAL OF SKIN HOMOGRAFTS

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Two previous publications[1,2] have outlined a critical experiment to assay the relationship of elements of the circulating blood to the survival of homografts of skin in the rabbit. It was found, if 1 cc. of homologous whole blood were injected 5 days prior to the implantation of a homograft of skin from the same donor, that such pretreatment enhanced the survival of the homograft, and this was true of second-set homografts as well. The active enhancing agent in homologous whole blood was demonstrated to reside within the erythrocyte after the red cells had been hemolyzed. When the erythrocytes had been tagged with radioactive chromium, it was found that their site of deposition was the lymph node in proximity to the site of injection, which was also near the homotransplant. Such deposition suggested that here might be the site of action of the enhancing agent.

As a result of these findings, the auricular lymph node was excised surgically, and a homograft of skin transplanted distally upon the ear. Transplants made soon after regional lymphadenectomy behave in a manner similar to that of enhanced grafts, and this was true of second-set as well as of first-set grafts. Enhancement following lymphadenectomy was in the magnitude of a factor of 2 for the first-set grafts. In addition, when only one auricular lymph node was removed and bilateral homografts applied to the ears, no enhancement of either graft was observed. When bilateral auricular nodes were removed, however, both homografts survived longer (FIGURE 1).

The regional lymph nodes that had been immunized were weighed to see whether there was an increase in mass following homotransplantation. Control weights of normal anatomical auricular lymph nodes averaged approximately 35 mg. There was a steady increase in the weight of the auricular lymph node, following homotransplantation, from the first day onward. Average weights upon successive days progressed from 60 mg. on the first day to 100, 115, 145, 190, 206, 252, and 307 mg. on the eleventh day. An increase in weight followed autotransplantation; however, the weights were less. A slight increase in weight also was observed for several days after a sham operation upon the anesthetized rabbit.

The case of an interesting human patient verified our laboratory findings concerning lymphadenectomy. In this case the life of a homograft transplanted upon the ipsilateral arm of a woman who had undergone radical mastectomy with removal of the axillary lymph nodes was prolonged by a factor of 2½ beyond that of the control homograft transplanted to the opposite arm, where the axillary lymphatics were intact.

The patient (M.R.) was operated on at St. Luke's Hospital on May 21, 1959, for lymphedema of the right arm that followed radical mastectomy for carcinoma of the breast. Presumably because of delayed wound healing and irradiation, surgical lymphedema became manifest in the treated arm, which became so cumbersome that the patient was referred for a plastic procedure to lessen its unwieldiness. A surgical procedure of the Macey type was carried out, consisting of total removal of skin, subcutaneous tissue, and fascia of the upper arm and forearm. The skin of the specimen was removed in strips that were sutured together and reapplied to the arm in the manner of a sleeve. In the medial aspect of the treated upper arm a 2.5-cm. homograft of male skin was transplanted upon a muscular bed, distal to the axilla from which the lymph nodes had been removed. On the corresponding area of the opposite normal arm, in which intact axillary lymphatics were present, a homograft of similar size and from the same donor was transplanted by identical technique (FIGURE 2*a* and *b*).

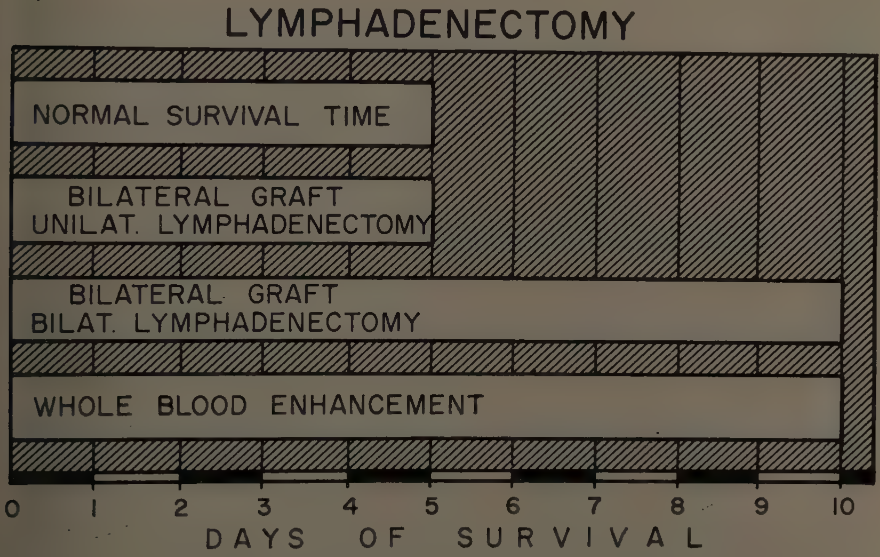

FIGURE 1. When bilateral homografts were transplanted to the ears of a rabbit, enhancement did not occur when one auricular lymph node only was removed. Bilateral lymphadenectomy would, however, produce homograft enhancement.

The surgical result was satisfactory, with complete success of the autografts. The homografts proved to be interesting. The control homograft showed evidence of necrosis by the seventh day and had completely disappeared by the twenty-second day. In the wake of its destruction, the circular peripheral incision contracted so as to become a dimpled scar. On the arm from which the lymphatics had been removed the homograft grew without evidence of necrosis until the fifty-fourth day, which was a prolongation in the life of the homograft of two and a halffold. Biopsies were made of both grafts upon the twelfth day, prior to the loss of either homograft. Hematoxylin and eosin sections showed merely a collection of inflammatory cells in the control, which presumably were to herald the reaction of rejection. Elastic-tissue slides failed to show rupture of elastic-tissue fibrils in either specimen at the time of biopsy.

Work by Scothorne[3] and by McGregor and Conway[4] and others has indicated that lymphatic anastomoses between graft and bed occur at approximately the fifth day, a time at which we noted histological as well as macroscopic evidence of homograft rejection. In our control series the first-set graft grossly appears half necrotic by the fifth day. A second-set graft transplanted on the fifth day was totally necrotic by the second day. We then varied the time intervals between the first-set and the second-set homografts

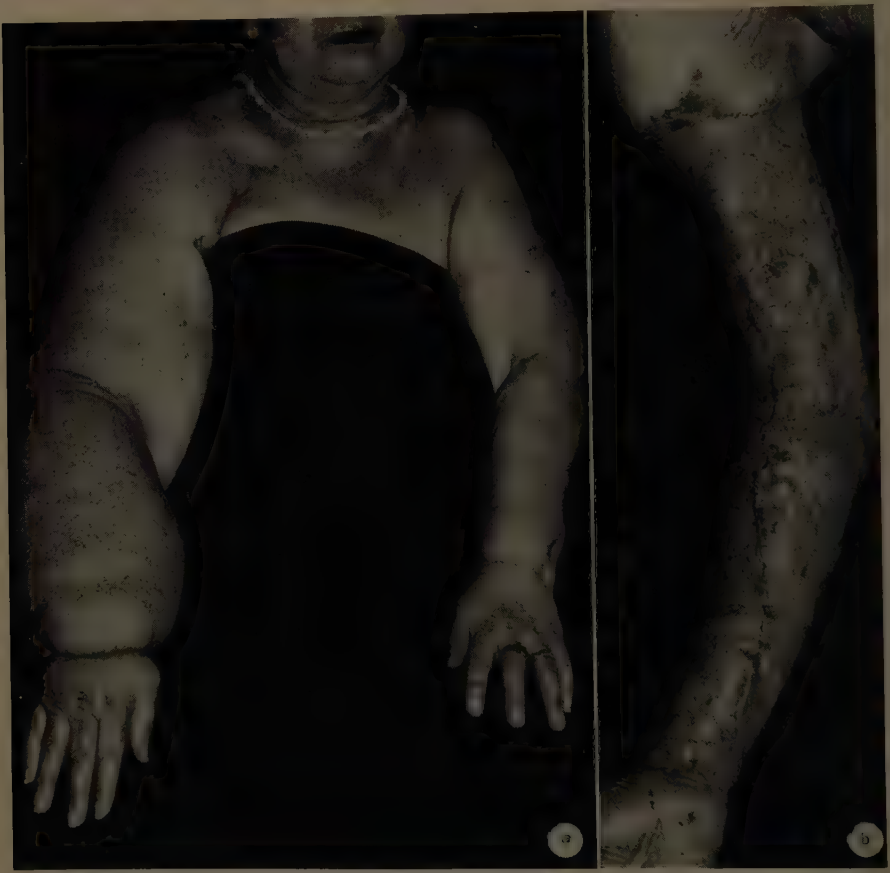

FIGURE 2. (a) A plastic surgical procedure of the Macey type was performed to alleviate the unwieldiness of the right upper extremity of a female patient who had developed surgical lymphedema following radical mastectomy for carcinoma of the breast. Beside coverage of the upper arm and forearm with autografts, a 2.5-cm. homograft of skin from a male donor was applied to the upper medial aspect of the arm, proximal to which the axillary lymph nodes had been removed. A control graft of similar size from the same donor was placed upon the same area of the opposite arm in which axillary lymph nodes were intact. (b) The survival of the homograft placed upon the treated arm from which axillary lymph nodes had been removed was enhanced by a factor of 2½ over the control.

to ascertain precisely upon which posttransplantation day the homograft immunity is established.

The time interval between implantation of the first- and second-set homografts was diminished so as to be less that five days. When this interval was reduced to two days, the second-set graft lasted for five days before it exhibited the typical half-necrotic, half-life loss. This was true as well for second-set grafts transplanted three and four days following transplantation of the first immunizing graft. However, if the interval of five days existed, when presumably the regional lymph node was immunized through lymphatic anastomoses, the second-set graft survived for only two days before it was completely lost. Pathologically, the second-set reaction appeared to be different from the dry gangrene of the first. Second-set grafts exhibited edema and ecchymosis, interpreted as resulting from vascular anastomoses followed by vascular rhexis with interstitial hemorrhage. Such phenomena have been well described by those working with transparent-tissue chambers.

It should be noted that in our second-set transplants few mononucleated cells were seen at the time that the second-set grafts were lost. This, however, does not mean necessarily that mononucleated cells may not participate in the destruction of the second-set graft. Their relative paucity may be accounted for by the early breakdown of circulation, so that quantitatively fewer mononucleated cells are transported to the bed of the graft, however, those present well may be involved in the reaction of rejection.

To test the hypothesis that immunization of the regional lymph node occurs only following lymphatic repair, several first-set grafts from the same donor were transplanted upon the rabbit ear. One first-set graft was left *in situ* for four days and then removed, when a second graft was implanted in an adjacent area; thus the two first-set grafts were *in situ* for a total of seven days. Second-set homografts transplanted to the opposite ear endured as first-set grafts, being half necrotic by the fifth day. This indicted to us that dosage is of importance in immunization only when repair of the lymphatic circulation occurs and that early interruption of such lymphatics fails to immunize the regional node.

Finally, impressed by the work of Mitchison,[5] attempts were made to effect a modified form of adoptive transfer (FIGURE 3). In this experiment a regional lymph node was immunized by a homotransplant placed upon the ear of a rabbit 5 days previously. The node was removed and cells of the node were suspended in saline. A cell count of this fluid revealed that it contained 375,000 lymphocytes per cubic centimeter. This suspension containing lymphocytes (presumably immunized) was injected variously into a new recipient, intradermally, subcutaneously, and intra-arterially. A homograft from the original skin homograft donor, who presumably immunized the node of the second rabbit, was transplanted to the recipient of the cell suspension, the third rabbit. A homograft from a fourth rabbit was placed on the opposite ear, as a control. In 10 experiments on 40 animals there was no indication that injections of lymphocytic suspension from presumed immunized nodes actually immunized the recipient. Instead, in each instance the homograft transplanted after the injection of the "immunized" suspension behaved as a first-set homograft.

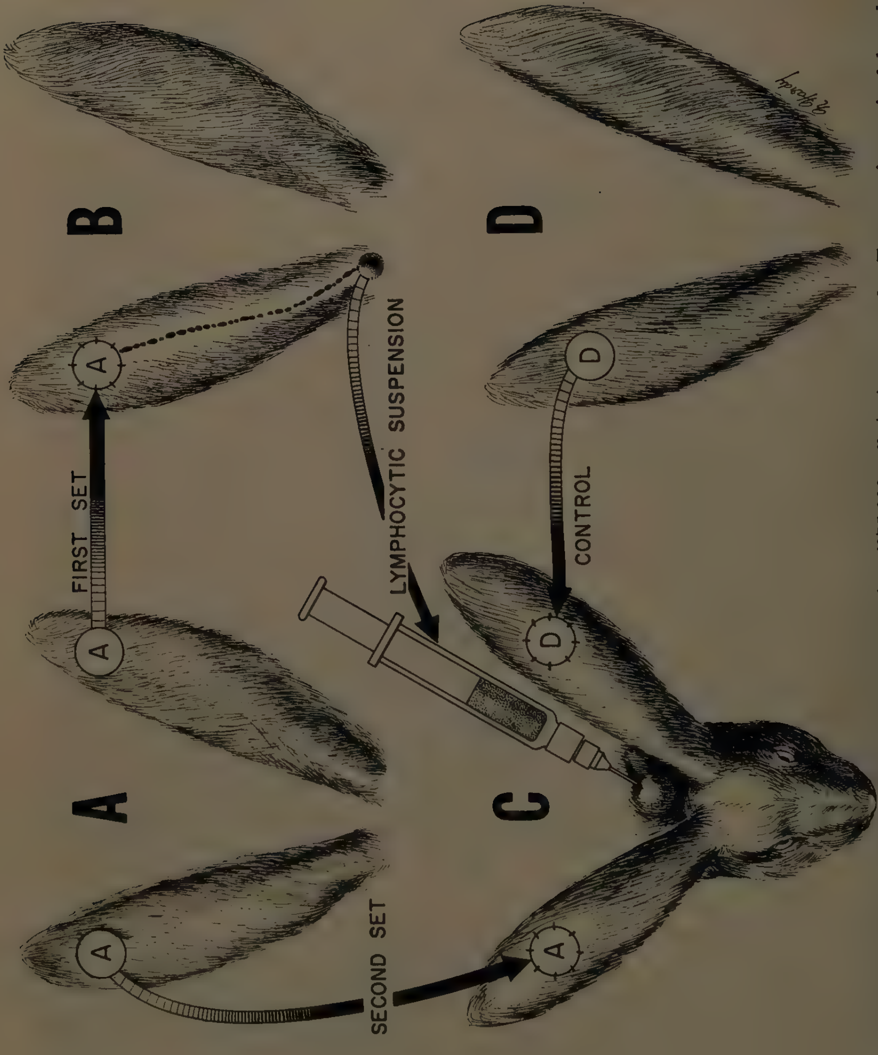

FIGURE 3. Lymphadenectomy in which a lymphocytic suspension (375,000 cells/cc.) was used. From an immunized lymph node, a type of adoptive transfer was attempted (to C). This passive transfer was challenged by a graft from the original donor (A) and controlled by a graft from a fourth rabbit (D). In no instance in 10 experiments was immunity transferred; all grafts to C behaved as first-set grafts.

References

1. Stark, R. B., H. Brownlee & R. P. Grunwald. 1958. Homologous whole blood as an agent for enhancement of skin grafts in the adult rabbit. A preliminary report. Ann. N. Y. Acad. Sci. **73**(3): 772.
2. Stark, R. B. & E. Dwyer. 1959. The enhancement of homografts of skin in the adult rabbit using elements of homologous whole blood. Surgery. **46**: 277.
3. Scothorne, R. J. 1958. Lymphatic repair and the genesis of homograft immunity. Ann. N. Y. Acad. Sci. **73**(3): 673.
4. McGregor, I. A. & H. Conway. 1956. Development of lymph flow from autografts and homografts of skin. Transplantation Bull. **3**: 46.
5. Mitchison, N. A. 1954. Passive transfer of transplantation immunity. Proc. Roy. Soc. London. **B142**: 72.

Discussion of the Paper

Barrett: This paper has made me think of two or three findings that might have some relation to it. In the first place, the illustrations showing grafts on the rabbit's ears have strongly suggested reactions referred to by some people as the "white graft."

I think that some contributors to this symposium have demonstrated that, as an intermediate immune reaction against a homograft, one can produce passively a locally resistant but not finally resistant state, and that it results in the so-called white graft. This seems to imply that the host is reacting against the graft not strongly enough to kill it, but strongly enough either to prevent or to inhibit the early vascular connections, leading to these observations.

I feel sure that inbred rabbits are not being used; if they are, I should like to know where they are obtainable. The white graft may be representative of a system where there may be many minor elements of immunogenetic incompatibility, but still not strong ones, and I was thinking about some work that seems to be closely related to some of the observations reported in this paper. Briefly, its authors have stated that when mice of various grades of genetic disparity were used, it was found that in the heterologously diverse system used in parabiosis some fluid material crossed the line of parabiosis as evidenced by the transmission of dye, a common test for parabiotic union, but that nothing much else went across; if, however, the animals were closely related, not only did these fluids cross the line of union, but so did some cells. Moreover, if the animals were isologous, then not only the fluids but many, many cells were transferred from one animal to the other.

I wonder if that could have some bearing on what has been described in this paper. In relation to the enhancement observed from use of the hemolysate, I can say only that this is simply one of those situations in this kind of experimental work where many of us are apt to find ourselves in disagreement. I do not think it is because of any real disagreement; I think it is because in this field of endeavor in the last few years we have succeeded in opening up for more objective appraisal many viewpoints, each of which takes into consideration a wide variety of technical approaches. I feel that we might be in the position of having just finished dropping a button box, and we have now been scrambling around on the floor for quite a while before obtaining a set of buttons that match. I do not think that these disparities necessarily mean final disagreement.

With the use of stromata, for example, we get only an induced resistance in the particular system that we use: it is a different system than that used by some other laboratories. Carefully washed stromata provide a perfectly good antigen for the type of induced resistance that we are reporting in this paper in these particular mice, and when we test the hemolysate, it does nothing. We can see no evidence of enhancement. This may be explained by the fact that our tumor is not that kind of tumor.

As to the final point made as regards the transfer of lymph nodes, I might mention an unpublished work that I have permission to cite. At the time Mitchison made his earliest observations on the transfer of lymph nodes to passive transfer immunity, his assistant was O. L. Dube.

Working in a nearby medical center, he came to my laboratory as the easiest available source of certain materials, the same materials used in the paper under discussion. I think you can observe in those materials an extraordinarily high degree of immunity if you express it in terms of the fact that the tumor will not take; Dube thought this degree of immunity and the qualities of this tumor would suit his experimental needs, so I gave him some animals and some tumor, and he tried to repeat this work. He came back to me, however, to report that he had not obtained any positive results.

Therefore he requested more material. When the last few months of his two-year term of military duty at this medical center had arrived, he became rather anxious because he had no results to describe in a paper. I have forgotten the exact conclusions he reached from all this effort, but I shall paraphrase them. From highly tumor-immune animals, he took the peripheral lymph nodes and transferred as many as 20 to 30 lymph nodes into a single recipient. He then tested the latter for tumor immunity against DBA-49 and it had no such demonstrable immunity, which agrees, of course, with what has been emphasized here.

Joseph E. Murray: I think this is an interesting application of the use of a condition seen in clinical circumstances, to test the results of a regional lymph node's function in skin homograft rejection. It interested me that Stark achieved prolongation of survival here. This is slightly in contrast to the results that Hume obtained several years ago using the dog and the kidney as the source of his graft material. As you might remember, he wrapped the kidney in a water-tight plastic bag, hoping to eliminate the regional lymphatic effect. He found under these circumstances that such a kidney was rejected just as rapidly as one that might not have been so protected from the influences of the regional nodes.

A question I desire to ask is this: did Stark test the circulating antibody against the red cell or the hemolysate and, if so, what were his results? If enhancement is predicated upon a circulating antibody, this would seem to be a rather interesting finding.

Stark's observations also seem to be somewhat at variance with other results, especially those of Berrian and McKhann, who recently published a paper in the *Transplantation Bulletin* reporting that they were able to induce an immunity to skin homografts by the topical application of a graft, one every day.

These investigators removed the homograft from its bed on day 1 and put another one in its place, removing the grafts in this manner for 5 successive days, thus eliminating any lymphatic attachment, so to speak. As I recall their results, they were still able to get an immune type of rejection every time.

Chase: I have one technical question to ask. Is this hemolysate the supernatant that results after removal of the stromata? If so, was it obtained by centrifuging from an isotonic solution?

Many points in the paper warrant attention. One aspect concerns the large amount of blood that was transferred. Was any assessment made of the effect of injecting small amounts of erythrocytes that would be comparable with the volume of white cells that were transferred? It might be supposed, for example, that either very large volumes of erythrocytes or very small numbers of white cells were being injected, and that the actual difference in result is not characteristic of the cell type itself.

Dorothy A. Madden (*Presbyterian-St. Luke's Hospital, Chicago, Ill.*): We have been engaged in work very similar to yours except that, instead of employing a skin graft, we have been using musculo-fascial transplants after transfusing blood. Our experience, I feel, may shed some light upon white-graft reactions, as we have made many histological studies of these musculo-fascial grafts and have found that the reaction one finds histologically two-weeks postoperatively, in comparison with a normal autograft and homograft, is not similar either to an autograft or to anything like the classic homologous reaction. Rather, there is a complete absence of inflammation if blood has been transfused from the donor of the graft before the graft is put into place—a complete absence of inflammation, usually a vascular penetration of the graft, and the graft then has lost completely its ability to proliferate. In addition, the host no longer seems capable of producing collagen, responses toward this graft or, if at all, only very minimal responses.

I ask Stark what the histological findings are of those homografts in which he has transfused blood prior to transplantation of the graft.

May I comment that we have been able to get consistently reproducible results by using as little as 1 ml. of blood as the transfusing agent.

Stark: A number of questions has been raised.

I do not truly believe that our grafts were similar to those described by others at this conference as white-graft reactions. We employed markedly diverse strains of rabbits, using New Zealand Whites, usually Flemish Giants and chinchillas. Many of the grafts in these animals, when peeled away from their beds, were vascularized. We often did this very early, and it did not seem to be a matter of a plasmatic type of circulation.

The point that Barrett brought up concerning the differences in response that he and Breyere and others have obtained in using the stromata of erythrocytes for immunity is, I believe, not really any different from the data we have described. It is a matter of dosage, and it certainly depends upon the animal used. When we attempted to inject daily for five days the amount of blood that we were using, we obtained immunity instead of enhancement. However, if we renewed the whole blood injections upon every fifth day only, we kept our grafts growing for a much longer period. Accordingly, there appeared to be a difference here in the response that could probably be attributed to dosage and to sequence of injection.

The question Murray brings up is a very good one, and I wonder if the plastic bag actually protects the kidney here, because all it does, I think, is prevent the peripheral invasion of inimical substances. Actually, the kidney is attached to the host vascularly, and all of the host's RE system is still intact.

We found no hemagglutinins in our experiments. We were using 30 per cent bovine albumin, and we made no titrations because we found no agglutination following a period of settling and then of centrifugation.

Regarding the amount of blood, I have nothing to add beyond what I have already said as regards the hemolysate that resulted from washing the erythrocytes. We then hemolyzed them, and finally spun them down to obtain the supernatant for use. The stromata also were used in a series of experiments.

CHASE: Did you centrifuge the stromata from isotonic solution?

STARK: No. We did this in distilled water.

CHASE: I recommend that you try the effect of using isotonic solutions. Swollen stromata will stay in suspension during centrifugation.

STARK: I am not too clear about the behavior of the musculofascial transplant. This is as a free graft, I presume, and, in general, all free grafts of muscles do not survive and are replaced by fibrous tissue. This is a general biological observation; accordingly I expect your microscopic findings in these grafts differ from ours. Ours was the classical histologic picture so well described by Dammin and others in the past.

IN VITRO STUDIES OF THE INTERACTION OF LYMPH NODE AND HOMOLOGOUS TISSUE*

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Introduction

Several independent lines of evidence from *in vivo* experiments point to the importance of the lymph nodes in the development of immunity to homologous tissue transplants. These are (1) the adoptive transfer of immunity to tumors (Mitchison, 1954) and to homologous skin (Billingham *et al.*, 1954); (2) the general similarity between the cytological changes found in the regional lymph nodes draining a skin homograft and those usually associated with the production of antibodies against bacterial and other antigens (Scothorne, 1957); and (3) the experiments of Weaver *et al.* (1955), using the millipore membrane technique, which indicate that "activated" lymphoid cells are essential to the mechanism of homograft destruction.

The importance of lymphoid cells—rather than serum antibodies of classic immunology—in the homograft reaction is emphasized further by the dermal hypersensitivity transfer reaction elicited in guinea pigs when "sensitized" cells from regional lymph nodes are injected intradermally into the donor of the immunizing graft (Brent *et al.*, 1958).

The present paper deals with the attempted production of a homograft response *in vitro*. In view of the evidence outlined above, one might anticipate no great difficulty in demonstrating *in vitro* the destruction of homologous tissue by lymph nodes from an immune recipient. In fact, however, there are at least four reports of failure to demonstrate the reaction *in vitro* (Harris, 1943; Medawar, 1948; Weaver *et al.*, 1955; and Trowell, 1959); and the present study makes the fifth. Two points perhaps should be emphasized. First, the repeated failure to demonstrate the homograft reaction *in vitro* in no way invalidates evidence from other sources bearing on the importance of lymphoid tissues in transplantation immunity; the failure means that we have not yet provided adequate *in vitro* conditions. Second, although the results of our experiments again—regrettably—are negative, we have defined more closely the conditions under which the reaction will *not* take place, and therefore have a better idea of what changes may be necessary to ensure that it will.

Material and Methods

Two strains of mice, CBA and Swiss white, were used. Skin homografts between the two were rejected promptly; in many cases graft destruction had already begun by day 6 after grafting, and in most was advanced by the day 8.

Full-thickness grafts of tail skin from CBA donors were transplanted to a site on the chest wall of Swiss white recipients, using the technique of Billingham and Medawar (1951). At various intervals after grafting the host animal was sacrificed and the "immune" ipsilateral axillary nodes removed and cut

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into fragments of about 2 mm. in greatest dimension. These fragments were then cultured in intimate side-by-side contact with similar axillary node fragments from the original donor of the immunizing graft.

The culture technique used was that of Trowell (1959) by which lymph nodes may be maintained in a healthy state and organized form for 6 days or longer. The medium was Trowell's protein-free liquid medium, T8.

After periods of culture of up to 6 days, the donor and recipient nodes, now more or less firmly united, were sectioned and studied microscopically. Cultures of nodes from autografted or unoperated Swiss white mice were set up as controls.

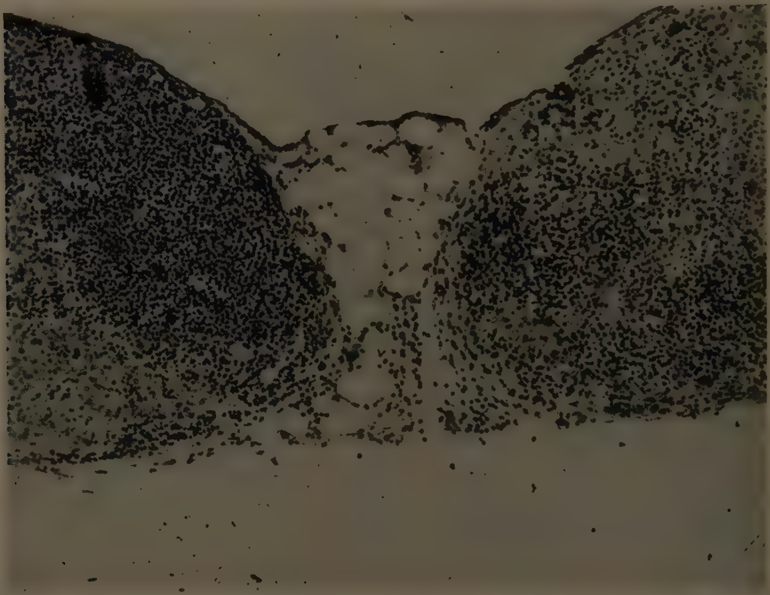

FIGURE 1. Combined culture of donor and recipient lymph nodes after 4 days *in vitro*. The recipient mouse received a skin homograft from donor mouse 4 days before sacrifice. ×125.

Results

Although full results will be published elsewhere, the following is a survey of the more important findings.

In general, the experimental material falls into two groups: one in which the recipient was sacrificed on day 4, 5 or 6 after grafting, that is, before the onset of graft destruction; the other in which the recipient was sacrificed on day 12 or 19 after grafting, after complete destruction of the graft.

FIGURE 1 shows the fusion that occurs between donor and recipient nodes after 4 days *in vitro*. There is a moderate loss of small lymphocytes from both fragments; this depletion, no greater than in controls, is due to the streaming of cells from the under surface of the nodes on to the paper-covered metal grid supporting them at the surface of the culture medium.

Most of the lymphocytes and reticulum cells on each side of the junctional zone appear healthy (as adjudged by the absence of nuclear pyknosis), as do the sparsely scattered lymphoid and reticulum cells that lie within the junctional zone. In fact, there are far fewer pyknotic lymphocytes at and around the boundary between the two fragments than there are at the centers of each fragment, where there has occurred the "central necrosis" not uncommonly found at the center of lymph node fragments in culture and attributed by Trowell (1952) to relative anoxia in those parts farthest removed from the oxygen supply.

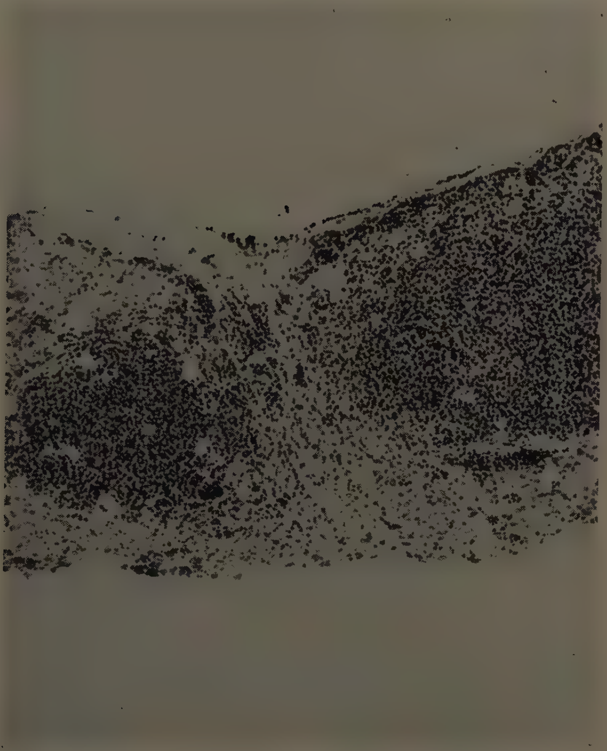

FIGURE 2. Combined culture of donor and recipient nodes after 4 days *in vitro*. Recipient received a skin homograft from donor 6 days before sacrifice. ×125.

More intimate contact between the 2 lymph nodes is shown in FIGURE 2, the photograph of a 4-day culture, taken on day 6 after grafting, when the graft was still alive, but slightly swollen and cyanotic. Serial sections show an extensive area of contact between the 2 fragments, each of which has presumably contributed reticulum and lymphoid cells to the boundary zone, although there is, of course, no way of distinguishing between donor and recipient cells. Again the cells at the boundary and, indeed, throughout each fragment appear healthy.

In 4-day cultures of nodes from animals that have reacted against and completely destroyed the immunizing grafts the appearances are similar. The degree of intimacy of fusion varies (FIGURE 3*a* and *b*), but in all cases the survival of lymphocytes in and about the boundary zone is as good as that found in controls.

Having failed to demonstrate an *in vitro* homograft response in 4-day cul-

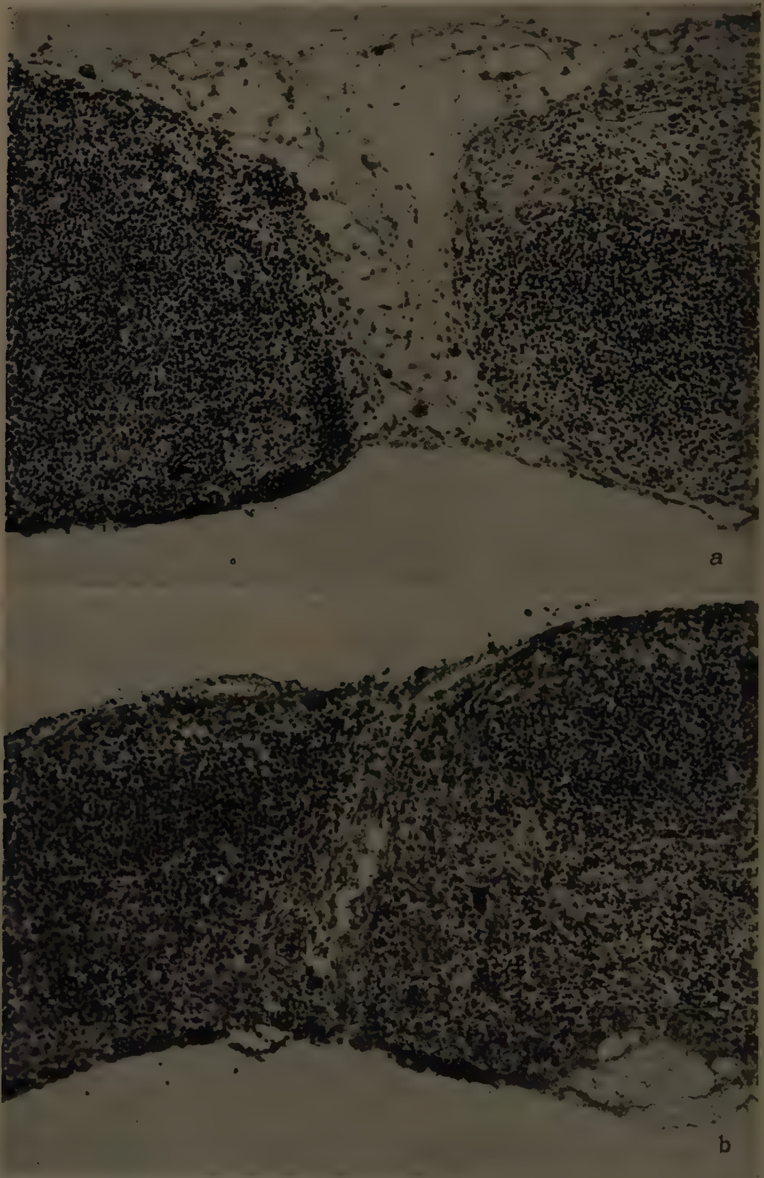

FIGURE 3. (*a*) Combined culture of donor and recipient nodes after 4 days *in vitro*; recipient received a skin homograft from donor 12 days before sacrifice. ×125. (*b*) Combined culture of donor and recipient lymph nodes after 4 days *in vitro*; recipient received a skin homograft 12 days before sacrifice. ×125.

tures, we extended the period of culture to 6 days. Here we feel less confident of the validity of our results. Many of the conjoined donor-recipient nodes showed moderate or severe lymphocyte destruction in one or both fragments but, since similar changes were found in many controls, we cannot ignore the possibility that they were the result of nonspecific technical deficiencies. This interpretation is reinforced by the finding of good lymphocyte survival in some of the recipient-donor combinations. FIGURE 4 shows a high-power view of an area close to the junctional zone from a 6-day culture set up, 20 days after completion of graft destruction. The few lymphocytes present are all dead, but the reticulum cells appear healthy, and one is dividing. Very good or excellent survival of reticulum cells was found in all cultures, even in those showing extensive lymphocyte destruction.

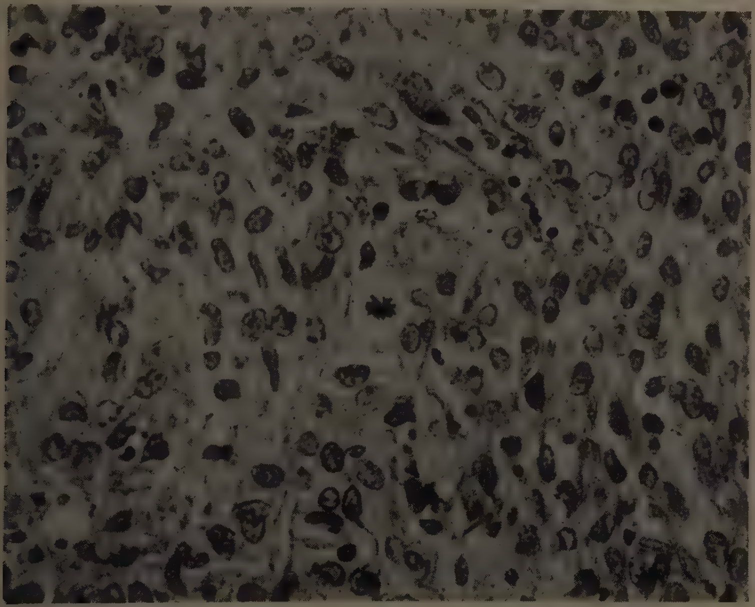

FIGURE 4. Combined culture of donor and recipient nodes after 6 days *in vitro*. Recipient received a skin homograft 28 days before sacrifice. High-power view of culture close to junctional zone. ×500.

Discussion

These results establish the fact that regional lymph node fragments from mice bearing skin homografts will coexist in intimate contact with lymph node fragments from the original donors for periods of 4 days in a protein-free culture medium. They suggest, but do not prove, that a homograft reaction is not effected under similar conditions even after 6 days.

These results confirm the previously reported failures to demonstrate the homograft reaction *in vitro* (Harris, 1943; Medawar, 1948; Weaver *et al.*, 1955; and Trowell 1959).

Harris (1943) was concerned mainly with the *in vitro* action of serum cytotoxins following heterotransplantation (mouse → rat), but referred briefly to the absence of cytotoxic effects in mixed cultures, in immune serum, of mouse tumor and lymph nodes from immune mice. He did not give sufficient information to allow a critical appraisal of his results in relation to our own.

The most extensive previous investigation of the problem is that of Medawar (1948). Using rabbits, he cultured fragments of lymph node or spleen from homografted animals, in intimate contact with skin from the original donor, and found no adverse effect on either mitotic or migratory activity of the cultured epidermis. Contact between recipient lymph node and donor skin was particularly intimate: in some cases the lymph node was completely invested by migrating epidermal cells. Such results were obtained even when "immune" serum was used in the medium. At the time these experiments were performed it remained to be established that the spleen plays a minor role, as compared with the regional lymph nodes, in the genesis of the immune response to orthotopic skin homografts. Most of Medawar's experiments involved spleen and not lymph node, and only one experiment with lymph node extended beyond the fourth day. In planning the present experiments we were able to take into account the subsequent clear demonstration (Billingham *et al.*, 1954) of the major role of the regional lymph node in the reaction.

The extensive experiments of Weaver *et al.* (1955) established the fact that tissue homografts survived when contact with host cells was prevented (by a millipore membrane), and suggested that homografts are destroyed by contact with "immunized" host cells, which they thought were probably lymphocytes. Their attempts to extend these results further by a demonstration of the reaction *in vitro* were, however, unsuccessful. Epidermal cells cultured with "immunized" spleen cells in plasma clots survived for 5 days. In analyzing the possible reasons for the failure of these *in vitro* experiments, the authors point out that, while many of the lymphoid cells were alive after 3 days, most were dead after 5 days of culture.

The most recent attempt to demonstrate an immune response *in vitro* is that of Trowell (1959), who showed, first, that such heteroplastic associations as rat lymph node and mouse ureter or mouse node and rat ureter survive normally for 6 days in culture and, second, that rat tissues could be cultured satisfactorily for 4 days in contact with lymph nodes from mice previously immunized by injections of rat tissue.

Profiting from the results of all these earlier attempts, we have tried to devise an experimental system of maximal sensitivity, paying particular attention to the following desiderata:

Optimal cultural conditions. The technique of Trowell (1959) provides by far the best available method of maintaining adult lymph node in prolonged health.

Maximal sensitivity of the "target" cells. If the homograft reaction is effected only weakly under *in vitro* conditions, it may be that the "targets" provided by Harris (tumor cells), and by Medawar and by Weaver and colleagues (epidermis) were sufficiently vigorous to override the reaction and survive. It was with this idea in mind that we used lymph nodes from the original donor as the target organ. As is well known, the homograft reaction, although individual-specific, is not tissue-specific, and an immune animal will react more rapidly against any of the tissues of the original donor and not merely against the original immunizing tissue. Adult lymphocytes are notoriously difficult to maintain *in vitro*; it seemed to us that even a weak homograft reaction should be enough to tip the scales against their survival.

As it turns out, however, it has not been possible to demonstrate an *in vitro* homograft reaction even by using what we believe to be a sensitive experimental method. We have found, it is true, extensive lymphocyte destruction at the boundary zone between the alien tissues after six days in culture, but there are cogent reasons for doubting that this destruction was in fact due to a homograft reaction: first, that similar appearances are found in controls and, second, that one would reasonably expect, from what occurs *in vivo*, the destruction of *all* cells in the immediate vicinity of a homograft reaction. We find, on the contrary, that the reticulum cells in the boundary zone are healthy, as judged by microscopic appearance and, what is more decisive, by the occasional evidence of mitotic division.

Despite these negative findings we are encouraged in the belief that under appropriate conditions it is possible to demonstrate the reaction *in vitro*, and that it is worthwhile to find out what these conditions are.

Summary

Fragments of regional lymph node from Swiss white mice carrying skin homografts from CBA donors do not exert any destructive effect when cultured *in vitro* in intimate contact with lymph node fragments from the original donor for four days.

Acknowledgment

We are grateful to G. M. Wyburn for his interest and encouragement and to Mary Johnston and D. Munro for their technical assistance. It is a particular pleasure to acknowledge our indebtedness to O. A. Trowell of the Medical Research Council Unit at Harwell for the generous training he provided in the use of his technique.

References

Billingham, R. E., L. Brent & P. B. Medawar. 1954. Quantitative studies on tissue transplantation immunity. II. The origin, strength and duration of actively and adoptively acquired immunity. Proc. Roy. Soc. London. **B143:** 58–80.

Billingham, R. E. & P. B. Medawar. 1951. The technique of free skin grafting in mammals. Brit. J. Exptl. Biol. **28:** 385–402.

Brent, L., J. Brown & P. B. Medawar. 1958. Skin transplantation immunity in relation to hypersensitivity. Lancet. **2:** 561–564.

Harris, M. 1943. The role of humoral antagonism in heteroplastic transplantation in mammals. J. Exptl. Zool. **93:** 131–145.

Medawar, P. B. 1948. Tests by tissue culture methods on the nature of immunity to grafted skin. Quart. J. Microscop. Sci. **89:** 239–252.

Mitchison, N. A. 1954. Passive transfer of transplantation immunity. Proc. Roy. Soc. London. **B142:** 72–87.

Scothorne, R. J. 1957. Studies on the response of the regional lymph node to skin homografts. Ann. N. Y. Acad. Sci. **64**(5): 1028–1039.

Trowell, O. A. 1952. The culture of lymph nodes *in vitro*. Exptl. Cell Research. **3:** 79–107.

Trowell, O. A. 1959. The culture of mature organs in a synthetic medium. Exptl. Cell Research. **16:** 118–147.

Weaver, J. M., G. H. Algire & R. T. Prehn. 1955. The growth of cells *in vivo* in diffusion chambers. II. The role of cells in the destruction of homografts in mice. J. Natl. Cancer Inst. **15:** 1737–1767.

SUPPRESSION OF TRANSFERRED LYMPH NODE CELLS IN NEONATAL RABBITS BY THE INJECTION OF LEUKOCYTES*

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Although tissue transplantation immunity has been most widely studied in the area of skin homografting, aspects of transplantation immunity have also been studied in a number of other systems, including recently the homotransfer of lymph node cells. Whereas in skin homografting the timing of rejection of the graft affords the measure of immunological response of the host, in the studies of lymph node cell transfer the measure of transplantation reaction of the recipient's tissues has been provided by the degree of interference with the functional activity of transferred cells in the production of antibody. The system of lymph node cell transfer in which tissue transplantation effects have been studied in this laboratory is summarized schematically in FIGURE 1. On the left side of the figure are shown the experimental situation and the kind of data obtained on simple transfer of lymph node cells in this system. Lymph node cells obtained from rabbits not previously injected with *Shigella paradysenteriae* are incubated *in vitro* with a *Shigella*-trypsin filtrate and transferred to homologous recipient animals. In the sera of the latter, agglutinins to *Shigella* can be found subsequently in a typical pattern of concentration versus time.[1] It was shown recently that, if the transfer of such cells was preceded, by about a week, by the injection of the prospective recipient animals with leukocytes obtained from the donors of the lymph node cells, agglutinins failed to appear in the sera of the recipients or did so in low concentration, as shown in the central section of FIGURE 1. This indicated that the injection of leukocytes prior to transfer had induced an immunological response to the transplantation antigens of the donor, and that this reaction had caused suppression of the production of agglutinins on subsequent transfer of lymph node cells.[2] It was shown later that this suppressive effect could be transferred passively by serum. If leukocytes were obtained from the prospective donors, pooled, and injected into other adult rabbits, the pooled sera of the latter, if injected into recipients together with lymph node cells, could cause suppression of the transferred cells, as indicated by the low concentration of agglutinin in the sera of the recipient animals.[3] This experimental design is shown in the last panel of FIGURE 1.

The present study grew out of an attempt to demonstrate in this system another effect that has been observed in tissue transplantation, namely, that of immunological tolerance. It was considered that, if a state of tolerance could be established in neonatal rabbits by the injection of rabbit leukocytes, this state might be reflected on subsequent transfer of lymph node cells from the adult to the young rabbits by agglutinin levels in the sera of the young recipient animals higher than those usually found after transfer. Such an effect

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could not be demonstrated; rather, it appeared that the injection of rabbit leukocytes into neonatal rabbits could actively induce the suppression of lymph node cells subsequently transferred. The data presented below were obtained in experiments on the active induction of this lymph node cell-suppressive effect in neonatal rabbits and in early attempts to transfer passively the lymph node cell-suppressive effect by the use of sera obtained from neonatal rabbits that had been injected with rabbits' leukocytes.

Lymph node cell transfer effect	Suppression of lymph node cell transfer effect Actively induced	 Passively Induced
	Donor leukocytes injected into prospective recipients 7 days before cell transfer	Leukocytes of 60 to 70 rabbits injected into other adult rabbits for production of rabbit antirabbit-leukocyte serum
Lymph node cells + *Shigella*-trypsin filtrate	Lymph node cells + *Shigella*-trypsin filtrate	Lymph node cells + *Shigella*-trypsin filtrate + Anti-leukocyte serum
Transfer to recipients	Transfer to same recipients	Transfer to recipients

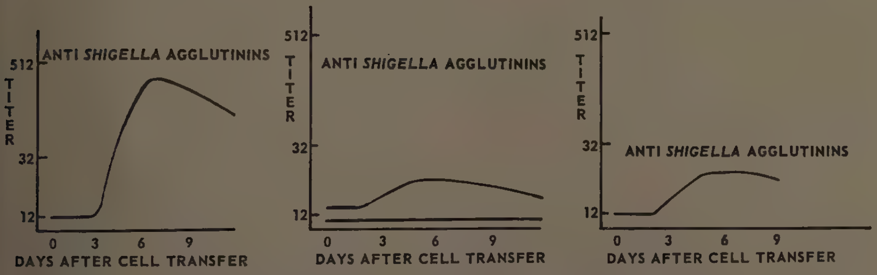

FIGURE 1. Experimental plan and typical results of transfer of rabbit lymph node cells incubated *in vitro* with *Shigella* antigen. Left, simple cell transfer; center, effect on cell transfer of active induction of host tissue reaction to donor leukocytes; right, transplantation reaction passively transferred to recipient animals by antirabbit leukocyte antibodies.

Experimental

Injection of rabbit leukocytes into neonatal rabbits: effect on transferred lymph node cells. As indicated above, these experiments were undertaken in an attempt to induce tolerance in neonatal rabbits to cells of other rabbits. Blood leukocytes from prospective donors of lymph node cells were injected into newborn rabbits and, 9 to 12 weeks later, lymph node cells were obtained from the adult rabbits, incubated *in vitro* with *Shigella* antigen, and transferred to the young rabbits. On subsequent examination of the sera of these recipients it was found that the agglutinin titers were not higher than those of control recipients; in fact, they were substantially lower, as may be seen in the upper part of TABLE 1. Thus, the recipients that had received the neonatal injection of donors' leukocytes had not only failed to show a greater degree of acceptance of transferred lymph node cells but gave evidence of a suppressive effect on such cells, presumably because of the leukocyte injection in early life.

In older recipient rabbits it had been found that a maximal degree of suppression of transferred lymph node cells was obtained between 7 and 20 days after the injection of recipients with donors' leukocytes.[2] Accordingly, in order to demonstrate more clearly in the neonatal rabbits a suppressive effect on transferred lymph node cells due to prior injection of leukocytes, a number of litters of newborn rabbits were divided into 2 parts. Rabbits of 1 part of each litter were injected, usually within the first day of life, with blood leukocytes from prospective donors of lymph node cells, and *Shigella* antigen-incubated lymph node cells were transferred from these donors to all the rabbits of the litter after a substantially shorter interval of time than that used in the earlier experiments. As may be seen in the lower part of TABLE 1, the agglutinin titers of recipients preinjected near birth with donors' leukocytes were markedly lower than those of their noninjected littermates, indicating that the neonatal injection of the leukocytes had induced a reaction of the homograft type against the donors' lymph node cells.

TABLE 1

EFFECT ON LYMPH NODE CELL TRANSFER OF INJECTION OF RECIPIENTS NEAR THE TIME OF BIRTH WITH LEUKOCYTES FROM THE PROSPECTIVE DONORS

Age of rabbits at time of:		No. of lymph node cells transferred ($\times 10^6$)	Maximum agglutinin titers of recipients		
			Injected with donor leukocytes soon after birth	Not injected with donor leukocytes	
Injection of donor leukocytes	Cell transfer			1-kg. recipients	Littermates of neonatal recipients
2 days	81	204	24	384, 384	
8 hours	75	206	64, 48, <12	512, 384, 192, 128	
10 hours	74	206	16	96	
1 day	60	206	64, 64, 32, 12	384, 192	
1 day	52	172	48, 32, <12, <12	512, 512, 128, 128	
1 day	43	222	<12		
1 day	36	172	96, 32, <12, <12, <12	256	
1 day	31	222	32, <12, <12		3072, 768, 512, 512, 384
10 hours	30	222	64, <12		2084, 768, 512, 512
3 days	12	200	12, 12, <12		384, 256, 256, 192, 192
1 day	8	175	<12, <12		
1 day	8	200	12+		3072, 384
1 day	8	182	64, 48, 32		1536, 1536, 1024
1 day	8	144	12, 12, <12		256, 128
8 hours	4	140	16, 16, 16		256, 128

In the course of studying the effect of injection of leukocytes into neonatal rabbits, the interval between this injection and subsequent cell transfer had been decreased from 2½ months to 1 month and even further, as can be seen in the lower part of TABLE 1. It was of interest to note that, in the control recipients of 11, 8, and even 4 days of age, substantial agglutinin levels developed. The question of whether very young animals can be used effectively as recipients of lymph node cells is the subject of some disagreement in the recent literature. Thus, Dixon and Weigle[4,5] have reported that neonatal rabbits are inadequate as recipients of lymph node cells, whereas Sterzl[6] and Holub[7] have effectively transferred homologous lymph node cells and lymph cells to neonatal rabbits, and Mitchison,[8] Trnka,[9,10] and Papermaster *et al.*[11] have done so in a chicken system. Since in the present study the transfer of lymph node cells to very young rabbits was followed by the appearance of agglutinins in the range of titer shown for the control littermates in the lower part of TABLE 1, further experiments not involving the prior injection of leukocytes were carried out in which the age of recipients of antigen-incubated lymph node cells was further reduced from 4 days to 1 day. It was found that agglutinins appeared in such recipients, usually by the fifth day, and reached a peak on the seventh day. The peak agglutinin titers were again of the same order as those shown for the control recipients in the lower part of TABLE 1. No attempt has been made here to compare the effectiveness per million lymph node cells in 1-kg. and neonatal rabbits. This point will be discussed elsewhere (Harris, Harris, and Farber, in preparation).

TABLE 2
AGGLUTININ TITERS OF NEONATAL AND 1-KG. RECIPIENT RABBITS INJECTED AT 2 LEVELS WITH DONOR LEUKOCYTES PRIOR TO LYMPH NODE CELL TRANSFER

Recipients	Geometric means of maximal agglutinin titers ($\log_2$) of recipients preinjected with donor leukocytes at 2 levels		
	10^7	10^5	0
Neonatal	3.2	6.9	8.3
1 kg.	2.5	6.3	8.0

Comparison of the active induction of the lymph node cell suppressive effect in neonatal and 1-kg. recipient rabbits. The effect of varying the numbers of donors' leukocytes injected was compared in neonatal and 1-kg. recipient rabbits. Rabbits of each age group were injected with 10^5 or 10^7 leukocytes 1 week prior to cell transfer. The geometric means of the maximal titers of each group after cell transfer are shown in TABLE 2. It may be seen that there was evidence of a substantial degree of suppression of transferred lymph node cells in recipients preinjected with 10^7 leukocytes and distinctly less suppression in those given 10^5 leukocytes. On comparing the effectiveness of the preinjection in rabbits of the 2 age groups, very little difference was found between the neonatal and 1-kg. rabbits, respectively, at either level of preinjected leukocytes, the slight difference in each case suggesting a somewhat greater degree of suppression in the 1-kg. recipients.

Effect on transferred lymph node cells of rabbit-antirabbit leukocyte serum produced in neonatal animals. The next series of experiments was directed at the question of whether the injection of rabbit leukocytes into neonatal rabbits would result in the production of antibodies that would be effective in the suppression of transferred lymph node cells, as had been found in the case of adult rabbits. Preliminary results of 2 kinds of experiments will be presented. In the first of these the prospective donors of lymph node cells were used as the source of leukocytes. Adult rabbits were bled, their leukocytes collected and pooled, and these were injected at a level of 50×10^6 into newborn rabbits. Nine and 11 days later the young rabbits were bled and serum was prepared from the pooled blood. Subsequently, the adult rabbits were sacrificed, and cells obtained from their lymph nodes were incubated with *Shigella*-trypsin filtrate, washed, and then incubated with given amounts of the serum produced in the neonatal rabbits. Such cells were transferred to irradiated 1-kg. rabbits, control recipients being given aliquots of cells that had not been incubated with the antileukocyte serum. In the other type of experiment blood was obtained from approximately 60 adult rabbits and pooled. Leukocytes obtained from such blood were injected in the same number into neonatal rabbits and into adult rabbits. Nine and 11 days later these animals were bled, and sera of rabbits of each age group were pooled. Such sera were tested for lymph node cell-suppressive effect by the procedure outlined above, the donors of the lymph node cells in this case bearing no relationship to the rabbits used as the source of leukocytes for injection. In each experiment in which such serum pools were tested the geometric mean of the maximal titers of control recipients was determined. For each group of rabbits given lymph node cells treated with a given volume of a given rabbit-antirabbit leukocyte serum the geometric mean maximal titer was also calculated, and the difference between the 2 means was determined. The results obtained

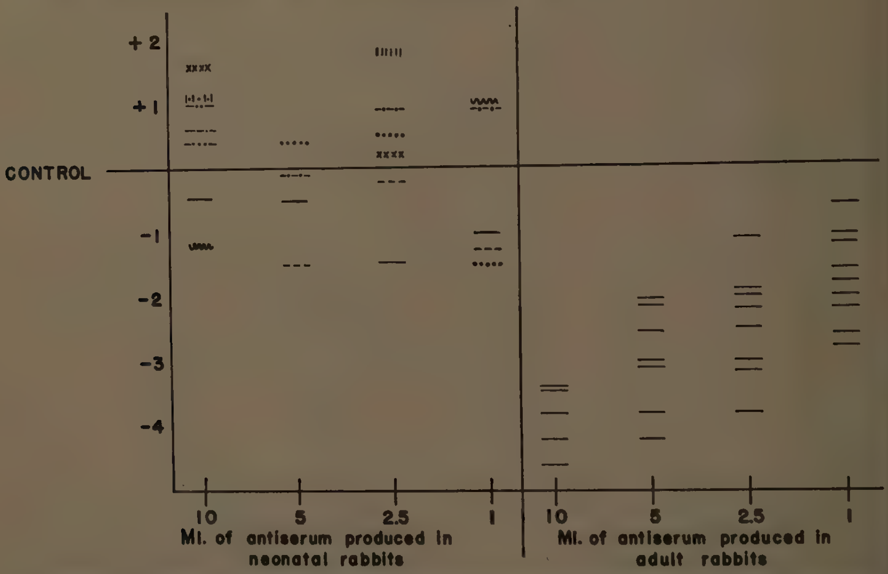

FIGURE 2. Effect on transferred lymph node cells of antirabbit leukocyte serum prepared in neonatal rabbits and adult rabbits, respectively. The horizontal line marked CONTROL presents a reference level, that is, the geometric mean maximal titer of control recipients in each experiment. The differences, in $\log_2$, between the mean control titer and the mean titer of recipients of lymph node cells incubated with various quantities of the rabbit-antirabbit leukocyte sera are shown for each group of recipient rabbits by the horizontal bars. The serum pools of the neonatal rabbits are marked individually.

on testing serum pools of both kinds are presented in FIGURE 2, where the differences are shown between mean maximal agglutinin titers of control recipients and those of recipients of serum-treated lymph node cells in the same experiments. It can be seen in FIGURE 2 that the rabbit-antirabbit leukocyte serum pools produced in neonatal animals by either procedure produced no consistent effect on the results of lymph node cell transfer at any of the amounts of antileukocyte serum tested. In contrast, the comparable pools of antileukocyte serum produced in adult rabbits caused a substantial decrease in anti-*Shigella* agglutinin production by the transferred cells when the sera were used in volumes of 10 ml., and decreasing degrees of suppression when used in progressively smaller amounts, as shown elsewhere.[3] In the case of the sera produced in neonatal rabbits by the injection of leukocytes, no difference in effect could be observed whether the leukocytes had been obtained from the prospective donors of lymph node cells or from larger numbers of other rabbits. Accordingly, data obtained with both kinds of serum are grouped together in the left side of FIGURE 2. The individual sera are identified, however, to show the scatter among the various sera of such differences as were found. Rabbit-antirabbit leukocyte sera were also prepared in 1-kg. rabbits and were examined for effectiveness in suppression of transferred lymph node cells. The data obtained with such sera thus far were not sufficiently extensive for inclusion in FIGURE 2. They indicated, however, that the sera produced in the 1-kg. rabbits were as effective in suppressing the cell-transfer effect as the analogous sera prepared in fully grown rabbits, or very nearly so.

Discussion

The data presented above demonstrate that the neonatal rabbits can react immunologically to tissue transplantation antigens from the first day of life. The ability of young animals to react to transplantation antigens could also be deduced from reports of 2 recent studies that involved the transfer of lymph node cells. Sterzl[12] injected rabbit spleen cells into 5-day-old rabbits and then injected *Brucella suis* into these recipients at various intervals after the transfer of the cells. Agglutinins to *Brucella* were subsequently found in the sera of the recipients only if less than 4 days had elapsed between cell transfer and the injection of antigen. Sterzl concluded that the splenic cells had not been able to survive more than 4 days in the tissues of the young recipient animal, presumably because of an immunological reaction of those tissues against the transferred cells. In the other study referred to, Trnka and Riha[13] incubated hen spleen cells *in vitro* with bovine serum albumin (BSA) and transferred these to embryonated eggs or to newly hatched chicks. After the hatching of the chick embryos anti-BSA was found in sera of these chicks, but not in sera of the chicks used as recipients after hatching. It was concluded that the failure of appearance of anti-BSA in the chicks used as recipients after hatching was due to the effects of a homotransplantation reaction in the period before the transferred cells could produce antiprotein antibodies, whereas the chick embryos were not capable of such reaction against the transferred cells. In the present study an immunological response of tissues of the neonatal rabbit to homotransplantation antigens was induced by prior injection of the donors' leukocytes. This injection was, in fact, almost as effective in the neonatal rabbits as in the usual 1-kg. recipient, as was found after the injection of leukocytes at 2 levels, rather widely spaced.

The effectiveness of antigenic stimulation of neonatal rabbits with homotransplantation antigens was of especial interest in this study, since in an earlier study a role in the suppression of transferred lymph node cells had clearly been demonstrated for antirabbit leukocyte antibodies, yet newborn rabbits have been found to be ineffective in the production of antibodies to many antigens (Sterzl[14] in the case of *Brucella*, Eitzman and Smith[15] working with BSA, and Harris *et al.* with bovine gamma globulin, and *Shigella*). It was in an attempt to clarify this issue that the production of antirabbit leukocyte antibodies was undertaken in the neonatal rabbit and, as was seen in the experiments reported above, it was not found possible to transfer the lymph node cell-suppressive effect by rabbit-antirabbit leukocyte sera prepared thus far in neonatal rabbits under the conditions used to obtain such sera in older rabbits. This situation is being examined further by the use of greater stimuli for the production of homologous antileukocyte antibodies in neonatal rabbits; for example, by the injection of rabbit leukocytes in greater quantities or with adjuvants. In view of the similarity in the degree of active induction of the lymph node cell-suppressive effect in neonatal recipients and 1-kg. recipients, the disparity in the effectiveness of antileukocyte sera obtained in these 2 groups of rabbits under the present conditions is as yet unexplained. On the one hand, this difference may be due to a quantitative difference in rates of antibody formation in the two groups. Thus a much lower rate of antibody formation in the neonatal rabbit would lead to poorly suppressive sera but might result in a disproportionately better degree of suppression when the lymph node cells have been transferred to the animal injected with the rabbit leukocytes, where a cumulative effect of the antileukocyte antibodies would be possible. On the other hand, this difference could indicate that in the neonatal animals an immunologic mechanism other than antibody formation may be involved in the actively induced suppression of lymph node cells. This problem is being approached currently in experiments on the production of the 2 effects in the 2 age groups of rabbits with the use of different levels of antigenic stimulation.

Summary

The injection of rabbit leukocytes into neonatal rabbits leads to the suppression of lymph node cells subsequently transferred to the young rabbits. Thus, if lymph node cells from the adult rabbits are incubated with *Shigella*-derived antigen and transferred to the neonatal rabbits, the expected agglutinins for *Shigella* fail to appear in the sera of the recipients.

In the induction of this effect given numbers of cells are almost as effective in neonatal rabbits as in 1-kg. animals.

Antirabbit leukocyte sera prepared in neonatal rabbits by the same procedure as had produced effective lymph node cell-suppressing sera in older rabbits were not found to have this effect on transferred lymph node cells.

References

1. Harris, S., T. N. Harris, C. A. Ogburn & M. B. Farber. 1956. J. Exptl. Med. **104:** 645.
2. Harris, T. N., S. Harris, C. A. Ogburn & M. B. Farber. 1958. J. Immunol. **80:** 308.
3. Harris, S., T. N. Harris & M. B. Farber. 1958. J. Exptl. Med. **108:** 411.
4. Dixon, F. J. & W. O. Weigle. 1957. J. Exptl. Med. **105:** 75.
5. Dixon, F. J. & W. O. Weigle. 1959. J. Exptl. Med. **110:** 139.
6. Sterzl, J. 1957. Folia Biol. (Prague). **3:** 1.
7. Holub, M. 1958. Nature. **181:** 122.
8. Mitchison, N. A. 1957. Folia Biol. (Prague). **3:** 72.
9. Trnka, Z. 1957. Československ. mikrobiol. **2:** 344.
10. Trnka, Z. 1958. Transplantation Bull. **5:** 73.
11. Papermaster, B. W., S. G. Bradley, D. W. Watson & R. A. Good. 1959. Proc. Soc. Exptl. Biol. Med. **102:** 260.
12. Sterzl, J. 1958. Transplantation Bull. **5:** 73.
13. Trnka, Z. & I. Riha. 1959. Nature. **183:** 546.
14. Sterzl, J. 1955. Folia Biol. (Prague). **1:** 193.
15. Eitzman, D. V. & R. T. Smith. 1959. Proc. Soc. Exptl. Biol. Med. **102:** 529.

Discussion of the Paper

H. S. Lawrence: There are several comments to be made on these interesting studies that Harris has presented.

One of the phenomena that seems to be described by Dr. Harris' results is the unique vulnerability of dissociated cell populations to the deleterious effects of serum antibody resulting in a type of homograft rejection.

There is evidence from studies on delayed allergy in man following transfer with cells or cell extracts indicating that whatever happens to activate or trigger the recipient cell population following transfer does so before the homograft rejection reaction has been called into being. The reason for believing this is the prolonged duration of sensitivity following cell transfer in man with whole cells or extracts. In support of this concept is Chase's more recent work in the guinea pig, showing that if special attention is given to the degree of sensitivity of the donor of leukocytes and the volume of cells used, the guinea pig begins to approximate man in respect of the prolonged duration of transferred delayed allergy. I think that in these specific instances the cellular transfer mechanism gets across whatever homograft rejection barriers do exist, and that it inaugurates in the recipient a process the expression of which has some similarities to active sensitization. Without getting into the matter too deeply, it is possible that our concepts of active and passive sensitization may be altered in the light of more recent information.

I wonder what might happen if Harris did an experiment similar to that which Siskind and Thomas report elsewhere in these pages and transferred his serum into a host that already possessed a transferred cell population in the act of producing antibody. The finding that tolerant animals do not produce antibody following cell transfer in the way one might expect they should, could be related to the fact that homograft sensitivity, rather than tolerance, was induced.

In fact, according to Harris' data on the "tolerant" animal injected as young embryo or as a newborn, the antibody titers resembled those of adult animals previously treated with an injection of whole cells.

Finally, a question and a statement. Was any cytotoxicity seen in the cells when they were incubated with serum before cell transfer? I should not be too disturbed over the fact that the quantity of serum utilized for passive transfer did not produce a good result in some experiments. The quantity of effective antibody in the recipient of passive transfer, might have to be much greater than that effective *in vivo.*

CHASE: The paper is a very complicated matter, and yet it represents a present principle of exceeding importance.

G. J. V. NOSSAL: There was one point mentioned, more or less in passing, which I think is of very considerable interest; this is the so-called Dixon and Weigle effect of the inadequacy, as they described it, of the neonate environment for the support of antibody-producing cells transferred from an adult donor.

We have investigated this rather carefully in both rats and mice and have come to the conclusion that, in this respect, animals seem to go through three stages of maturation. In the first stage (in which Dixon and Weigle made their investigations) the animal is incapable of producing antibodies itself and is incapable of passively supporting antibody production from normal homologous adult cells.

In the second stage (the stage with which Harris, Trnka, Sterzl, and Hrubesova from Czechoslovakia are dealing) the animal is incapable of forming antibodies itself, but is capable of supporting antibody production from transferred adult homologous cells. The third or final stage, of course, is when the animal can both support antibody production and form antibodies itself.

The very interesting point that we noted in our studies, not in different species of animals but actually in different strains within one species, is that animals may pass through these three stages at very different times, and the actual age of the recipient animal is absolutely critical. For example, in the Wistar rats that we have used, the period of change-over from Stage 1 to 2 was at approximately the third or fourth day of life, as it was in the Hall Institute strain of mice. But in C3H mice, this change-over from Stage 1 to 2 was achieved within quite a few hours after birth. In certain respects, therefore, neonatal C3H mice are more mature than Hall Institute mice of the same age.

I think it would be well to keep in mind the Dixon and Weigle effect as a very real phenomenon, but one requiring rather special care to demonstrate it in a given circumstance.

HARRIS: In the first place, with respect to Lawrence's comments, we have always considered this type of side issue concerning the possible suppressive effects of serum given to rabbits that already have cells in the process of forming antibody. We have done something more or less similar to this in giving serum to rabbits that were receiving lymph node cells from antigen-injected donors where the cells were as much as within a day of producing detectable antibody, and this suppressive effect is still demonstrable. We are preparing a publication on this now.

As to cytotoxicity, we have found agglutination of blood leukocytes and lymph node cells as transferred by such sera, but we have not done the cytotoxicity tests that are more commonly performed with dye uptake.

Actually, these newborn animals can do something in the way of producing antibody. If, for example, five times as much is given to them in the way of cells, that is, leukocytes, to induce antibody formation to the leukocytic antigens, they will nevertheless produce sera that are less effective when titrated out than the sera obtained in adults; there is such a marked disparity on the quantitative level that, although we have not worked it out as yet for presentation on a quantitative basis, it is clear that there is a difference in the degree of contribution of the two serum titers on the suppression of lymph node cells.

The question of humoral antibody formation also is a function of the antigen used. There is a wide variety of times at which rabbits can begin to be immunizible or react in the humoral antibody sense.

In so far as Nossal's question is concerned, we show here the result of transfer only to rabbits as young as 4 days old because the nub of the matter was to show what happens to rabbits that have had time to react to neonatal injections of leukocytes; it takes 3 days for that immunological action to occur after the neonatal injection of leukocytes. Although we had chosen 4 days as the time of cell transfer, we also carried that critical period on down to 1 day after or to the day of birth, thus working on 4-3-2-1-0 days in a varying number of studies of the day chosen for cell transfer to determine the most effective day. The animals are still competent to have antibody formed in their tissues on transfer of such cells and, of course, Dixon and Weigle's work was successful also with rabbits. There could not be strain differences as substantial in these rabbits as among mice. I wish we had strains of rabbits—of course, we have not—but at least these are all heterogenous populations which naturally represent different contributions of different stocks in different cities from various parts of Pennsylvania. These could be regarded by some as different strains of rabbits.

Chase: These matters are very complex, and yet they will form the substance of investigation for some years to come. No matter how a new fact is clothed because of the peculiarity of a particular system in which it is first discovered, the principle will eventually come to general appreciation. We may expect this area of concern to recur in future homotransplantation conferences.

SOME PECULIARITIES OF THE SURVIVAL TIME OF SKIN HOMOGRAFTS

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Introduction

There is no doubt now that the immunological process is the basis of tissue incompatibility. The intensity of many immunological reactions, as is well known from classic bacteriology, depends on the dosage of a foreign antigen. It is quite clear that antigen dose and immunological reaction have the same relation in transplantation immunity. The experiments of Medawar[1,2] in 1944 and 1945 with rabbits and of Lehrfeld and Taylor[3] in 1953 with rats showed that the survival time of small skin homografts was greater than that of larger ones.

We were interested in continuing this work. We wished to study in particular the recipient's reaction to the transplantation of big masses of donor tissue.

The interesting data obtained by Voronzova,[4] who experimented with large homografts of cold-blooded animals, were of great help to us in our work.

Experimental Study

White rats were used in our experiments. Most were males, 100 to 150 gm. in weight, and not related to each other. Transplantation was made under sterile conditions. We transplanted full-thickness skin homografts to the subcutaneous muscular layer. The survival times were determined from the clinical status and the circulation of blood in the grafts. The transplanted skin grafts were of different sizes (TABLE 1).

Homografts 0.25 to 1 sq. cm. in size were placed on 61 rats, homografts 2 to 6 sq. cm. were placed on 58 rats, and homografts 9 to 18 sq. cm. were placed on 22 rats. Finally, we transplanted skin 30 to 60 sq. cm. in size to 31 animals.

The survival times of the homografts of the first group of animals were 2 to 3 weeks, those of the second and third groups not more than 2 weeks. However, in the fourth group the results were different: of 31 grafts, 15 showed a longer than usual survival, 11 were viable for 1 to 1.5 months, and 5 for more than 2 months. We also had several animals on which homografts were viable during a period of over 8 months.

We have found in the literature some analogous data of Terasaki *et al.*,[5] who noted that larger skin homografts of chickens remained viable longer than small skin grafts.

FIGURE 1 illustrates the above-mentioned phenomenon. FIGURE 1*a* shows a second-group rat 2 weeks after the operation. The homograft became black and dry, and perished. FIGURE 1*b* shows a successful big skin graft on a rat of the fourth group 8 months after transplantation. The skin was so grafted that the fur grew in a direction opposite to that of the surrounding fur. A successful homograft begins to acquire sensibility 3 or 4 weeks after operation.

Histological examination (FIGURE 2) of the homografts showed that the transplant took well with the tissues of the recipient. It preserved the structure of normal skin.

We were interested in the proportion of homograft area to total skin surface. Since a rat weighing 100 to 170 gm. has a surface (skin) area of 110 to 180 sq. cm., our large transplants occupied one third of it.

TABLE 1
SURVIVAL TIME OF RAT SKIN HOMOGRAFTS OF DIFFERENT SIZES

Group no.	Graft size (sq. cm.)	No. animals	Survival (no. of animals)			
			Up to 14 days	Up to 21 days	From 30–45 days	More than 60 days
1	0.25–1.0	61	41	20	—	—
2	2–6	58	58	—	—	—
3	9–18	22	21	—	1	—
4	30–60	31	15	—	11	5

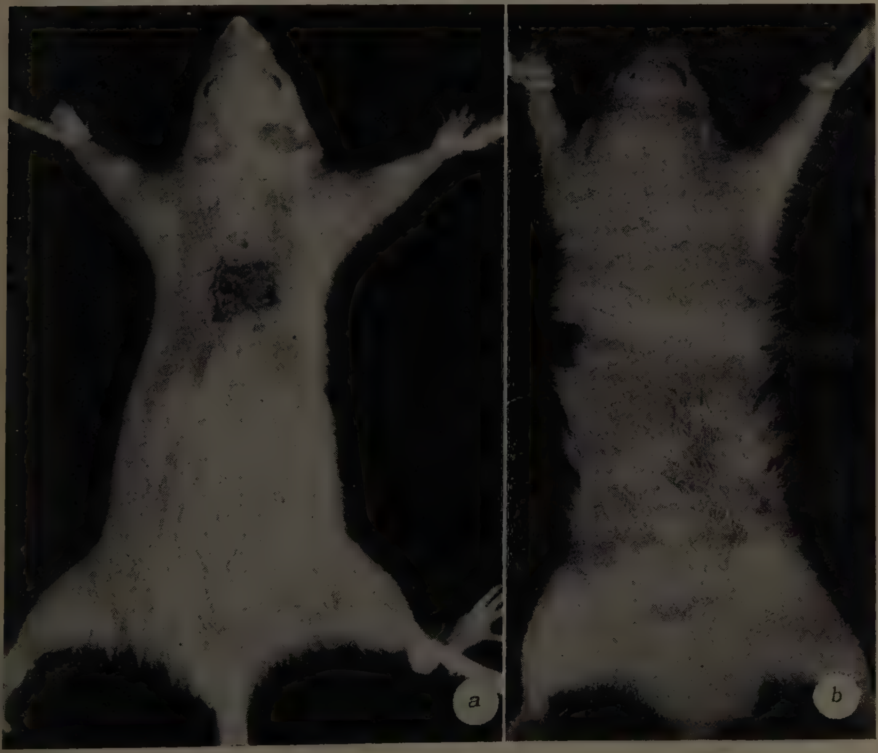

FIGURE 1. (*a*) Small homograft on rat 2 weeks after operation; the homograft has withered and died. (*b*) Large homograft on rat 8 months after operation; the homograft has survived.

The dependence of homograft survival time on size is illustrated in FIGURE 3. The horizontal axis shows the size of homografts in square centimeters; the vertical axis, the maximal survival time of the transplant after operation. It is seen that the survival time of the small skin graft is 3 weeks. Homografts 2 to 18 sq. cm., however, survive only 2 weeks. A homograft that is approximately one third of all the skin on the animal will last much longer.

What are the causes of this peculiarity of the larger skin homografts?

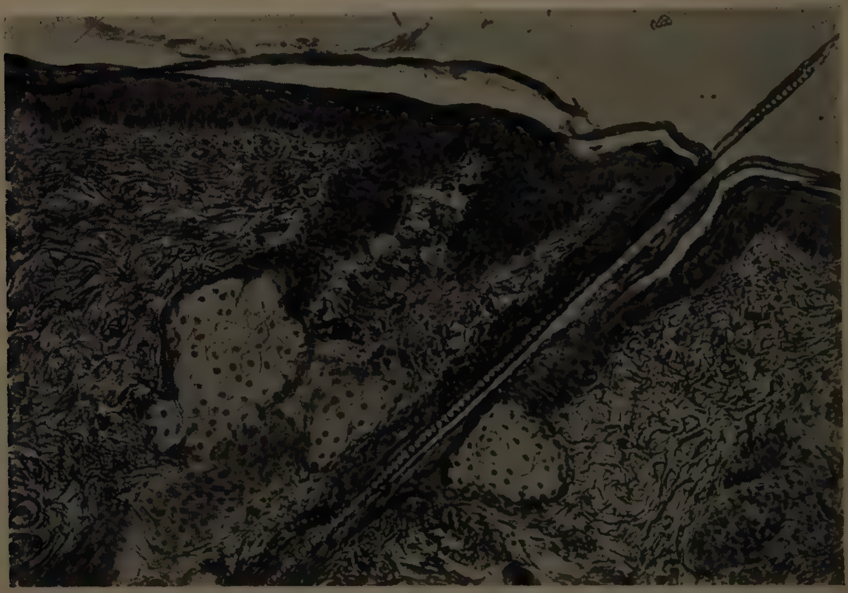

FIGURE 2. Successful homotransplant on rat. ×126.

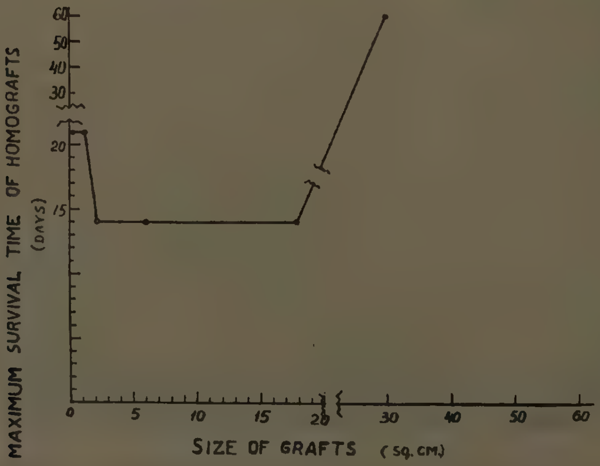

FIGURE 3. Dependence of survival time of homografts on their sizes.

To answer this, we thought it necessary first to study the role of the trauma to the recipient caused by an extensive transplantation. Here it is important to note that death of the animals soon after operation was common. In our experiments we placed a large skin autograft and, at the same time, a homograft of several square centimeters on one animal—a method of simultaneous homo- and autotransplantation of skin grafts (FIGURE 4). The homograft is put in the middle of the big autograft on the animal's back and fixed with sutures. After the initial take of the autotransplant the homograft is tested.

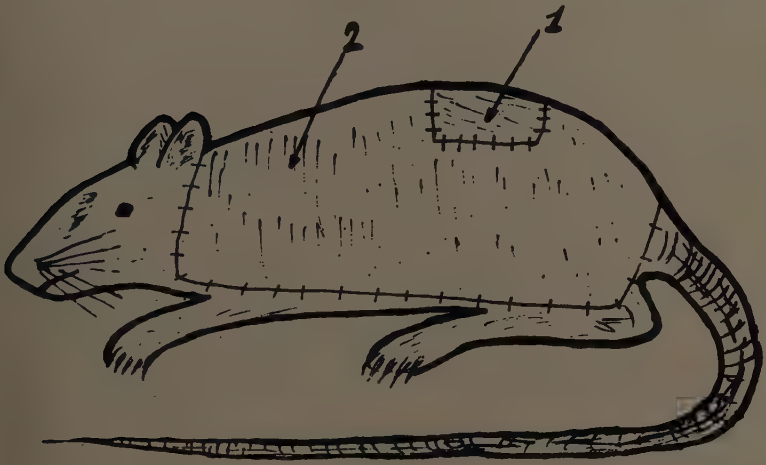

FIGURE 4.

TABLE 2
SURVIVAL TIME OF RAT SKIN HOMOGRAFTS TRANSPLANTED SIMULTANEOUSLY WITH LARGE SKIN AUTOGRAFTS

No. animals	Survival (no. of animals)			
	Up to 14 days	From 14–30 days	From 30–45 days	More than 60 days
25	17	6	1	1

As may be seen in TABLE 2, 17 of 25 homografts perished within 2 weeks after transplantation, 6 were viable for 3 to 4 weeks, and 2 were viable for longer periods of time.

Thus the transplantation of a large autograft prolongs the survival of a smaller homograft. It may be that the trauma inflicted during autotransplantation temporarily suppresses the reactivity of the recipient. However, in comparing these facts with the results of the large skin homotransplantations one may note that the survival times of the latter were longer than those of the smaller homografts surrounded by autotransplanted tissue. Evidently, it is not only the trauma to the recipient that plays a part in prolonging the life of a big skin homograft.

We then turned our attention to the animals' immunological reactions in cases of large skin homotransplantation. The experiments were made on rabbits because we have found that these animals are particularly useful in the study of such reactions: as we have shown,[6-9] rabbit recipients produce antibodies after the homotransplantation of skin. This peculiarity was used as a test of reactions to skin homografts of different sizes. On the tenth to twelfth days after transplantation we tested the serum of the recipients for hemagglutination (TABLE 3).

The data of TABLE 3 show that 8 of 12 recipients having homografts of 10 to 15 sq. cm. produced antibodies with titers of 1:2 to 1:32. Only 2 of 10 recipients having homografts of 200 to 300 sq. cm. produced antibodies. The titer of antibodies was not more than 1:4. From these data it appears that in rabbits the transplantation of big skin grafts leads to the suppression of immunological factors, in particular the production of antibodies.

The ability of large homografts to survive differed from that of small ones. The small skin grafts of the 12 recipients (TABLE 3) were rejected 7 to 9 days after transplantation. Although 6 big skin homografts broke down within the same period of time, 4 were viable longer. In their appearance and feel they were similar to the autotransplants until the end of the second week. Furthermore, the similarity was confirmed histologically two weeks after operation. The big skin homografts having survived well, we were greatly surprised by the death of the animals.

TABLE 3
ANTIBODY PRODUCTION AFTER HOMOTRANSPLANTATION OF RABBIT SKIN GRAFTS OF DIFFERENT SIZES

No. animals	Graft size (sq. cm.)	Antibodies		Antibody titer
		Detected	Not detected	
12	10–15	8	4	1:2–1:32
10	200–300	2	8	1:4

FIGURE 5*a* shows a small rabbit skin homograft 12 days after operation. At the place of the homograft there is only leukocyte scab. Epithelium of the host skin is growing under the scab. In FIGURE 5*b* is seen a large homograft 12 days after operation. The transplant has maintained all the elements of skin structure (epithelium and hair follicles). The host tissues do not appear to be forcing off the graft tissues.

It is possible that transplants of small size, which have a little dose of foreign antigen, produce a slighter transplantation immunity than do skin grafts of larger size. A very large skin graft, however, occupying about one third of the entire skin surface perhaps contains a massive dose of antigen, and immunization of the recipient by such a massive dose leads to the suppression of the defensive immunological factors and thus ensures a longer survival.

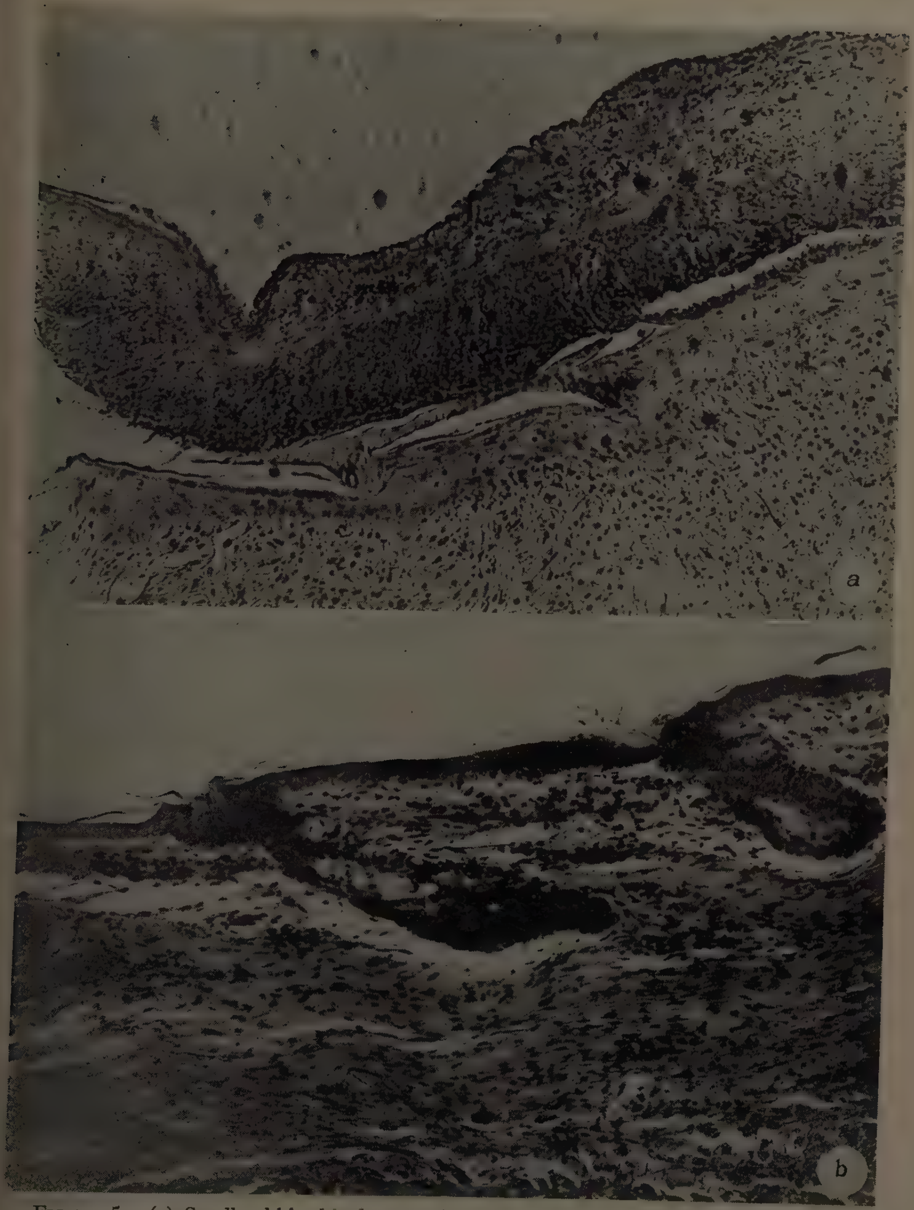

FIGURE 5. (*a*) Small rabbit skin homograft 12 days after operation. ×126. (*b*) Large rabbit skin homograft 12 days after operation. ×126.

Conclusions

A skin homograft occupying not less than one third of the skin area of an animal survives longer than does a small skin graft.

The trauma to an animal caused by the homotransplantation of a large piece of skin, while influencing the survival time of the graft, is not the chief factor in prolonging it.

The suppression of immunological reactivity by massive doses of foreign antigens evidently is the cause of longer life in the large homograft.

References

1. Medawar, P. B. 1944. The behaviour and fate of skin autografts and skin homografts in rabbits. J. Anat. **78:** 5; 176-199.
2. Medawar, P. B. 1945. A second study of behaviour and fate of skin homografts in rabbits. J. Anat. **79:** 1; 157–176.
3. Lehrfeld, J. W. & A. C. Taylor. 1953. The dose phenomenon in rat skin homografts. Plastic and Reconstruc. Surg. **12:** 432–438.
4. Воронцова. М. А. 1945. К вопросу о регенерации органов, состоящих из тканей различного происхождения. Бюллетень Московского общества испытателей природы. т.50 вып.3–4 стр.31–45.
5. Terasaki, P. I., W. P. Longmire & J. A. Cannon. 1957. The effect of size and donor age on survival of skin homografts in newly hatched chicks. Transplantation Bull. **4**(3): 112.
6. Зотиков. Е. А. 1956. Изменения иммунологических свойств сыворотки кроликов при гомотрансплантации кожи. Бюллетень экспериментальнои биологии и медицины. Ио. 7 стр. 58–61.
7. Зотиков. Е. А. 1957. Сравнительное изучения гуморальных и некоторых морфологических изменений, наступающих при гомотрансплантации кожи. Бюллетень экспериментальной биологии и медицины. г. Ио. 12, стр.91–95.
8. Зотиков. Е. А. 1958. От специфичности гуморальных факторов трансплантационного иммунитета. Бюллетень экспериментальнои биологии и медицины. г. Ио. 11, стр.72–77.
9. Зотиков. Е. А. 1959. О значении величины гомотрансплантированного лоскута кожи для продолжительности его жизни. Бюллетень экспериментальнои биологии и медицины. г. Ио. 2 стр.119–121.

Discussion of the Paper

Converse: I think it is possible to correlate Zotikov's observations in rats with some of the findings that we can observe in burns in clinical patients.

Over the years, those of us who treat burns have observed that patients who undergo extensive areas of skin loss maintain their homografts for a number of weeks, as a rule, before they are rejected. This is in sharp contrast to small experimental skin homografts of which we at New York University have made quite a large series in humans; in the latter, one never obtains a postoperative survival of more than 10 to 14 days.

The commonly accepted cause of this long-term survival has been attributed in the past to the physiological upset, or to an upset of the immunological response mechanisms, perhaps explaining why the burned patient tolerates the skin homografts for a longer period of time.

In view of Zotikov's observations, however, I believe that we must revise our previous thoughts somewhat and think of the response of the burned patient in terms of the effect of the actual dosage of antigen as having a direct effect on long-term survival. I think this is perhaps in keeping with what one sees in orthodox immunology, where the size of the dose of the antigen, the route of administration, and the individual species responses of the animal all act as factors that produce various types and degrees of immunological responses.

ROGERS: As a recent visitor to the Soviet Union, I have myself inspected the few animals that Zotikov describes, in which these massive homografts have apparently survived for a very long period of time. There is one important aspect of these findings by Zotikov and his associates that I propose to discuss briefly. In Moscow, Vladimir Demikhov has been publicized recently for his transplantation of major organs or organ systems, including that of the two-headed dog. Demikhov partially explained to me the longest 30-day survival of one of his two-headed dogs on the basis of Zotikov's findings in rats, namely that a massive amount of hypothetical antigen (supplied by the large amount of tissue such as the extra donor dog's head and forelegs might provide) overwhelms or exhausts or suppresses the host's immunological mechanisms to such an extent that the host dog cannot respond adequately.

In the presence of all the immunologists present, I feel a little hesitant in raising the term "immunoparalysis" in reference to Zotikov's findings, but the semantic connotation of this term has been argued by eminent immunologists at previous conferences here and, therefore, I believe that my reference to this term is not without precedent. This immunoparalysis, or whatever name it may assume in the future, in 5 of the 31 animals described by Zotikov, resulting in the survival for more than 60 days of massive skin grafts 30 to 60 sq. cm. in size, has implications that I am sure will be raised in the future whenever other surgeons are confronted with the prolonged survival of organs or larger masses of tissue, as Demikhov has been. There will certainly be those who will draw upon Zotikov's experiences as partial explanation of these prolonged survivals, especially if they take place in the absence of complicating factors affecting survival such as irradiation, uremia, severe trauma, adrenocortical "stress" phenomena, etc.

However, one should also possibly look to other explanations and factors accounting for some of these more recent long survivals of large amounts of homotransplanted tissue, especially in the experiments of Demikhov. When C. C. Guthrie, now living in Missouri, twice performed the "two-headed" dog operation with temporary success in 1908 (which he described in the J.A.M.A. of that year), he did not have at his disposal the ingenious blood-vessel suturing apparatus that Demikhov uses to give a very accurate and very rapid anastomosis and restoration of blood flow between the donor organs or "extra head and forelegs" and the host's blood stream. In addition, surgical techniques including pre- and postoperative management have improved markedly since 1908. Thus it is possible that the longer temporary survival of some of these large organ homotransplants in the Soviet Union and in the United States at present, might be explained in part by the availability of better surgical techniques and apparatus.

One cannot ignore, however, the unquestionable factor of immunological mechanisms being involved in the results of Demikhov and Zotikov. There are those, including Leslie Brent and H. S. Lawrence, who suggest in their excellent comprehensive reviews on homotransplantation, that immunological paralysis closely resembles the phenomenon of acquired tolerance: both phenomena would appear to be long-lasting, specific, and characterized by a long-term persistence of the antigen. Lawrence, in his *Physiological Reviews* paper, briefly summarized the recent immunologic experiments that support the hypothesis, which states that in immunological paralysis the available antibody is neutralized by the tremendous and excessive amounts of antigen as rapidly as the host forms it. When one considers the vast amount of polyantigenic material emanating from skin homografts equivalent to a third of the body surface of a rat or from the multiple types of cells and tissues present in a homografted extra head and pair of forelegs, it is easy to understand if this hypothesis is valid how the host could be temporarily or perhaps, in some cases, even permanently exhausted in its attempts to manufacture enough antibody to neutralize the excess antigen.

Both Brent and Lawrence suggest that a test for the presence of a state of immunologic paralysis (or immunologic unresponsiveness) might be the adoptive transfer of lymph node or spleen cells obtained from other specifically sensitized normal animals to see if this state of immunological paralysis can be abolished in the host animal. It might be worthwhile, therefore, if Zotikov were now to adoptively transfer such lymph nodes to those rats who, after many months, are still retaining the massive skin homografts, to see if such a transfer would bring about their rapid rejection. In Demikhov's animals, one might expect, if immunologic unresponsiveness or immunoparalysis is present, for adoptive transfer to possibly bring about a more rapid or immediate or acute rejection of the extra head and forelegs, rather than the slow, chronic edematous walling-off of the extra head that I personally observed last year in the "two-headed dog" with the longest survival, to date, of thirty days.

SOME STUDIES ON THE ANTIGENICITY OF CARTILAGE*

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With the possible exception of corneal transplants, cartilage appears now to be the only normal tissue whose cells persist indefinitely following homologous transfer to a genetically different host. Review of the literature on cartilage transplantation reveals that considerable speculation and difference of opinion concerning the fate of homotransplanted chondrocytes existed until 1957. During the last two years, however, Gibson,[5] Craigmyle,[3] and Schatten[10] have answered many of the questions concerning survival of homologous cartilage cells by reporting a continuous uninterrupted uptake of radioactive sulfur by transplanted chondrocytes. In addition, Peer[8] has shown recently that chondrocytes with female chromatin spots persist and can be recognized indefinitely in male recipients. These reports have confirmed earlier impressions formed by observing normal staining characteristics of living chondrocytes in fresh, unfixed cartilage homografts. The results of other experiments performed by Dupertius[4] and Schatten[11] suggest strongly that growth occurs in animal transplants, particularly in costochondral junctions; growth in a human cartilage homologous transplant, however, has not been demonstrated.

Bacsich[2] has postulated that the chondroitin sulfuric acid matrix that surrounds the cells in a cartilage graft may exert a protective influence. He has likened this defense to the mucopolysaccharide capsule that surrounds certain pneumococci and protects them from destruction by circulating antibodies. Bacsich's theory that a matrix barrier protects the chondrocytes is especially attractive in view of mounting evidence that the foreign tissue rejection phenomenon is an immune response of the delayed hypersensitivity type. Thus, if the tissue rejection antibodies are macromolecular proteins attached to large monocytes, hyalin matrix, which readily permits a diffusion of fluid and gases, might protect embedded cells completely by preventing cellular contact with antibody-carrying host cells. Recent experiments showing indefinite survival of homologous cells protected by an artificial millipore filter strongly suggest that such a mechanism is mechanically possible.

If the hyalin matrix of a cartilage transplant provides a mechanical barrier to cellular antibodies, it then becomes apparent that a cartilage chimera offers a splendid opportunity to study some of the characteristics of cellular antigens and antibodies. If the chondrocytes contain an antigen that is soluble in tissue juices or is free to diffuse through the matrix as particulate matter, these antigens should pass into regional lymphatics and immunize the host against other cells from the same donor animal. Following such an immunization by a cartilage transplant, the animal should then show an acceleration of the rejection of other tissues from the same donor animal. Such a rejection should be of the "second-set" type. Conversely, if the antigen is not free to diffuse

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through condroitin sulfuric acid matrix because of its molecular size or permanent cellular attachment, unprotected tissue grafts (such as a free skin graft) would be rejected as a first-set rejection. If the animal was found not to be immunized by the cartilage transplant, the mechanical defense mechanism commonly attributed to the intracellular matrix must apply to the release of antigen as well as the return of antibody and therefore cannot be used as evidence for antibody blocking unless chondrocytes can also be shown to persist in animals rendered immune by repeated grafting of other tissues such as spleen or skin. We have attempted to answer some of these questions by performing the following experiments.

Methods

Twenty C3H strain mice were implanted with cartilage grafts from 20 C57 strain mice. The graft consisted of the entire xiphisternum, which was transferred to the subcutaneous tissue of the abdominal wall of the host animal after it had been meticulously cleansed of all surrounding connective tissue. Ten cartilage grafts were transferred immediately and 10 grafts were fixed in formalin prior to transplantation. The formalin-fixed grafts were transplanted in an attempt to learn whether nonliving transplants were antigenic. The 20 C3H strain mice that received the cartilage grafts were subsequently challenged with 1.5-square-cm. full-thickness skin grafts from the same C57 strain donor mouse that had provided the cartilage. Half of the mice were challenged 2 weeks after they received cartilage, and the rest were challenged 4 weeks after the cartilage transplant. In order to determine a typical first- and second-set rejection time for this experiment, ten C3H mice were grafted with 1.5-square-cm. full-thickness skin grafts. Three weeks later they were challenged with a second skin graft taken from the same donor. All of the grafts were placed on the dorsal surface of the thorax, and the animals were housed in separate cages to prevent destruction of the graft by a companion.

A second experiment was designed to overcome, if possible, the theoretical objection of a low dose of antigen in tissue as relatively acellular as cartilage. Even though the xiphisternum grafts were relatively enormous grafts, being comparable to a graft weighing 60 gm. in a human being, we were able to obtain larger grafts by utilizing a rabbit-to-mouse transfer. In this experiment, cartilage was removed from the rabbit's ear—a technique that provided grafts 20 times larger than the xiphisternum grafts. These grafts filled the entire subcutaneous space under the anterior abdominal wall and were equivalent to a graft weighing approximately 1200 gm. in a 135-lb. human being. Ten mice that received massive grafts were challenged 4 weeks following the cartilage graft with skin grafts from the same rabbit that donated the cartilage. First- and second-set rejection times for this experiment were determined by an additional control experiment in which 2 sets of skin grafts were transferred between rabbits and C3H strain mice.

A third experiment was designed to evaluate the ability of skin antibodies to penetrate hyalin matrix and affect the viability of transplanted chondrocytes. In this experiment, chinchilla gray rabbits were made the recipient of 2 successive skin grafts from New Zealand white rabbits. Immunity to the donor rabbit was assured by demonstrating an unmistakable second-set rejection of the last skin graft. At the height of the immune reaction following removal of the graft, a 1 × 1 × 1 cm. cartilage graft was removed from the ear of the chinchilla gray rabbits and was transplanted to the subcutaneous tissue of the New Zealand white rabbits that had been successfully immunized by repeated skin grafts. In addition to the homologous cartilage, a piece of known dead cartilage preserved in Merthiolate was transplanted to be used as a control tissue for subsequent sulfur uptake studies.

Five hundred μc. of radioactive sodium sulfate (S^{35}) was injected intraperitoneally into each of the host animals. The rabbits were injected between 3 and 8 weeks following the cartilage graft. Between 18 and 24 hours following the injection of radioactive sulfur the rabbits were sacrificed and 3 pieces of cartilage were removed. The transplanted dead cartilage and the transplanted homograft cartilage were removed from their subcutaneous pockets, and a piece of autogenous cartilage was removed from the ear.

Extraction of sulfur from the cartilage was based on the work of Carius[12] and Young.[13] Specimens of cartilage were introduced into a Carius bomb with 5 to 10 mg. of sodium bromide and 1 to 2 ml. of red fuming nitric acid. Following digestion at 300° C. in a Carius furnace, the contents of the bomb were evaporated and 95 per cent ethanol and 1 to 2 ml. of benzidine hydrochloride reagent were added. The precipitate was centrifuged, suspended in ethyl alcohol, and recentrifuged. The precipitate was then evaporated to dryness in an electric heater. Following cooling in a desiccator, the precipitate was weighed and the radioactivity determined in a scintillation counter.

The Carius oxidation benzidine sulfate method is well suited to the measurement of radioactive sulfur in biological materials because the benzidine salt provides such a uniform deposit that the detection of radioactivity is considerably more accurate than for barium sulfate. By comparing the radioactivity of the sulfur extracted from autogenous, homologous, and dead heterologous cartilage, it was possible to distinguish living from dead cartilage.

A fourth experiment was designed to measure the ability of human plasma proteins to diffuse across the hyalin matrix of an *in vitro* slice of human cartilage. The diagram in FIGURE 1 depicts the mechanical arrangement utilized for this determination. The chamber on the left side of the Lucite cell contains tissue culture medium No. 199 (revised by Healey and Parker, 1953[7]) to which 6 per cent human gamma globulin has been added. It is separated from the right chamber, which contains only the tissue culture medium without proteins, by a 1-ml.-thickness slice of fresh human cartilage. In other experiments, fresh human serum was placed in the left chamber, and a bicarbonate-buffered salt solution was placed in the right chamber. The large amount of albumin in contact with the cartilage when serum was used was abnormal and, therefore, undesirable in our opinion. The osmotic effect of the proteins was partially balanced by raising the height of the protein solution to 140 ml. A mixture of 95 per cent oxygen and 5 per cent CO_2 was bubbled through the right chamber. A magnetic stirrer in both sides and the effect of the bubbling gases in the right side assured proper mixing.

A cathode-ray oscilloscope was used to measure any difference in potential between the 2 chambers that might develop secondary to the effect of the magnetic stirrers and salt solutions. If there was any difference in potential between the 2 chambers, it was of a magnitude of less than 200 μv. The protein-free solution is slightly acid, which causes the phenol red (added as an

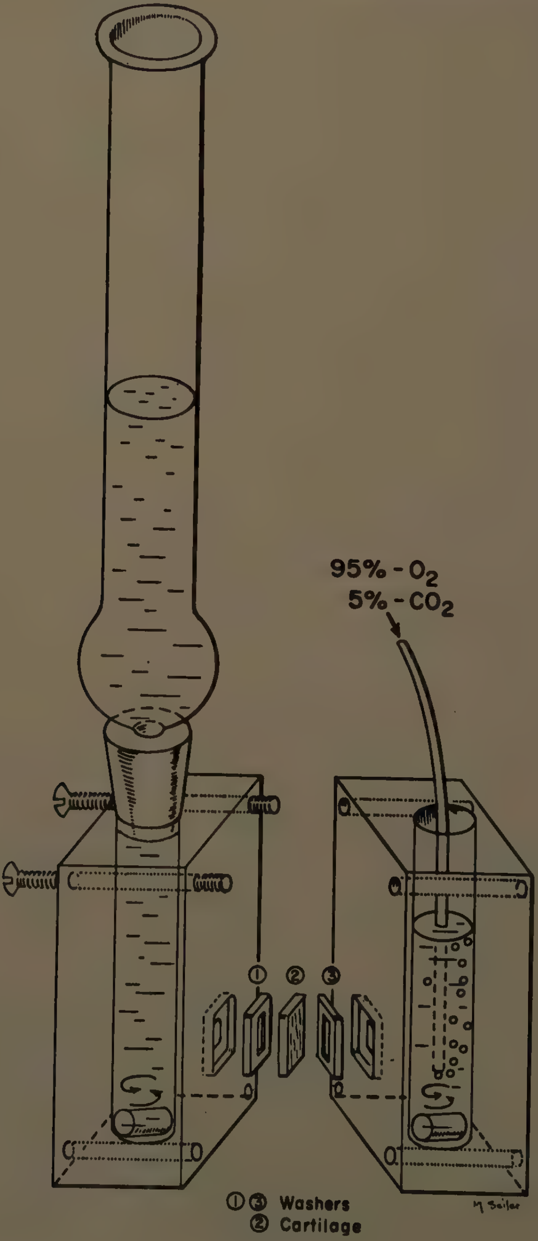

FIGURE 1.

indicator) to produce a yellow color. The protein-supplemented solution was slightly alkaline due to the effect of the buffered gamma globulin, and this caused the phenol red to produce a salmon pink color. As the salts and water diffused across the cartilage, the indicator turned pink on the right side, and the *p*H adjusted to approximately 7.2 on both sides.

Every hour, for 48 hours, 0.2 cc. of the fluid from the right chamber was removed. These samples were subjected to a precipitin test in which Coombs antihuman serum was utilized to show the presence of human protein. A 10^6 dilution of the 6 per cent gamma globulin solution could be detected with certainty by this method.

Table 1
Skin Rejection Times*: Control Groups
Number of Days Survival

No.	Experiment I		Experiment II		Experiment III	
	C57 mouse to C3H mouse		Rabbit to mouse		Rabbit to rabbit	
	1st set	2nd set	1st set	2nd set	1st set	2nd set
1	7	4	7	5	8	6
2	7	4	7	4	7	4
3	9	6	7	5	9	6
4	8	5	8	5	9	6
5	9	5	8	5	8	5
6	9	6	7	5	7	4
7	8	6	8	5	8	6
8	8	5	7	5	8	6
9	8	5	7	4	9	6
10	7	5	8	5	8	5
Av.	8	5.1	7.4	4.8	8.1	5.3

* The difference between the first- and second-set rejection times in all 3 experiments is statistically significant.

Results

Table 1 summarizes the control experiments for rabbit-to-mouse, mouse-to-mouse, and rabbit-to-rabbit first- and second-set skin rejections. The end point in these experiments was a sudden change from a pliable, soft texture to a stiff, leathery condition. Such an end point was quite definite and was relatively sudden in animals as genetically diverse as those selected for these experiments. We found this end point considerably more reliable than separation of the grafts, which could be influenced by the activity of the mouse and the type of fixation. None of the grafts became infected, and all of them took initially.

The first-set skin grafts in the C57 to C3H strain mouse control group had an average survival time of 8 days. The shortest survival time was 7 days, and the longest surviving graft remained viable for 9 days. The second-set grafts were applied as soon as all of the inflammatory reaction from the first grafts had disappeared and the wound was completely healed. The second-set grafts persisted for an average of 5.1 days. The longest surviving second-set graft remained viable for 6 days. The longest surviving second-set graft did not last as long as the shortest surviving first-set grafts; thus first-set and second-set rejection times for this experiment were clearly defined. Control experiments for the rabbit-to-mouse skin transfer in the second experiment revealed an average time of 7.4 days for the first-set rejection and an average time of 4.8 days for the second-set rejection. An average time of 8.1 days for the first-set rejection and an average time of 5.3 days for the second-set rejection were found in the rabbit-to-rabbit skin grafts (TABLE 1).

All of the animals that had received preliminary cartilage grafts rejected their challenging skin grafts in a typical first-set rejection time (TABLE 2).

TABLE 2
SKIN REJECTION TIMES*: CARTILAGE TRANSPLANT GROUPS
Number of Days Survival of Challenging Skin Graft

No.	Experiment I	Experiment II
	C57 mouse to C3H mouse	Rabbit to mouse
1	8	7
2	9	8
3	8	8
4	8	8
5	9	7
6	7	7
7	8	8
8	8	7
9	9	8
10	8	8
Av.	8.2	7.6

* The difference between these results and the second-set rejection times in TABLE 1 is statistically significant.

There was no difference in the rejection rate of animals that had received fresh small grafts, formalin-fixed grafts, or massive, fresh, rabbit ear grafts. Rejection of all the skin grafts followed a typical first-set rejection time.

The dead cartilage grafts had an average radioactivity of 176.2 cpm, which should be considered the background count for this experiment (TABLE 3). The homologous and autogenous specimens had counts that were significantly above the radioactivity in the dead cartilage, and are presented as evidence of the viability of the chondrocytes in these specimens. An elevated count in the homologous grafts probably reflected our inability to clean autogenous cartilage of surrounding connective tissue as thoroughly as the homografts that could be removed cleanly from their subcutaneous pockets.

We also attempted to demonstrate viability of the xiphisternal cartilage grafts in the mouse-to-mouse experiments. The results from these experiments were spread over such a wide range, however, that they were not statistically significant. The inaccuracy of these counts undoubtedly reflected our inability to obtain clean specimens of transplanted xiphisternal cartilage.

For this reason we utilized the rabbit-to-rabbit experiments when it was critically important to determine the viability of chondrocytes. The relatively massive grafts that could be used in a rabbit-to-rabbit experiment were easy to clean properly and submit to a sulfur extraction.

The precipitin tests performed on the aliquots of salt solution in the fourth experiment were consistently negative for human protein. Human cartilage does not permit the diffusion of human plasma proteins under the conditions of this experiment.

TABLE 3

S^{35} UPTAKE BY HOMOLOGOUS CARTILAGE TRANSPLANT IN IMMUNE RABBITS*

No.	Weeks after transplant	Counts per minute per 100 mg. dry wt.		
		Dead	Homologous	Autogenous
1	3	218	626	—
2	4	176	490	486
3	5	142	480	290
4	5	232	539	485
5	6	180	510	305
6	6	210	550	290
7	7	120	440	360
8	7	150	460	250
9	8	160	410	340
10	8	174	445	420
Av.		176.2	495.0	358.44

* The difference between the autogenous and homologous cartilage specimens and the control specimens of dead cartilage is statistically significant.

Discussion

Conclusive evidence attesting to the indefinite persistence of chondrocytes in homologous cartilage transplants has accumulated during recent years. Loeb[6] called attention to the prolonged persistence of homotransplanted chondrocytes and felt that it was evidence of a "low tissue specificity." Bacsich and Riddell,[1] attempting to explain the mechanism of persistence of chondrocytes and corneal cells, advanced the theory that similar mucoproteins in the matrix of cartilage and the ground substance of the cornea served as a protective armor against host antibodies. Whether such protection is a mere mechanical barrier to the macromolecular cellular antibodies currently believed to be responsible for homograft rejection or whether there is a more complex chemical destruction of antibodies is unknown. Peer has pointed out that chondroitin-sulfuric acid in the matrix of hyalin cartilage and mucoitin-sulfuric acid in the intercellular substance of the cornea are chemical isomers and that chemical analysis and staining reactions indicate that the mucoproteins of cartilage and cornea have much in common.[9] Whether the protection afforded tissues rich in mucoproteins is mechanical, chemical, or immunological, this protection is believed to be a defense against tissue antibodies. Our experiments, in which we demonstrated living rabbit cartilage in rabbits sensitized against rabbit skin, strongly suggest that the chondrocytes were protected from cytotoxic antibodies against the antigens in skin although, admittedly, it has not been proved that chondrocytes would be susceptible to these antibodies if they were not protected by a hyalin matrix.

In addition, the results of our first and second experiments indicate that, if chondrocytes carry a common tissue specificity antigen, it is physically contained within the hyalin matrix and apparently never reaches the reticuloendothelial system. The results of these experiments could be otherwise explained on the basis of an insufficient dose of antigen to elicit a second-set skin rejection or on a failure of chondrocytes to contain antigenic material that will produce foreign tissue antibodies that are the same as, or which cross-react with, epithelial antigen and antibodies. An attempt to rule out the dosage factor was made in the rabbit-to-mouse transplants, but it is conceivable that an even higher dose might have given an antibody response. The possibility that chondrocytes do not carry a tissue specificity antigen could not be eliminated using whole cartilage grafts.

That the hyalin matrix of a cartilage homograft is physically capable of preventing large molecules, such as gamma globulins, from reaching the chondrocytes is suggested by the failure of protein molecules to diffuse across sections of human cartilage in the *in vitro* diffusion experiments.

Summary and Conclusions

The rejection of C57 strain mouse skin by C3H strain mice that have been grafted previously with cartilage from the same C57 strain donor follows a typical first-set rejection pattern. Massive rabbit cartilage grafts transferred to C3H strain mice also failed to immunize the recipients against rabbit skin from the same donor rabbit.

Homologous cartilage grafts transplanted into rabbits rendered hyperimmune by repeated skin grafts from the same rabbit that donated the cartilage show an interrupted uptake of radioactive sulfur.

Plasma proteins in normal human plasma do not diffuse readily through *in vitro* slices of human cartilage.

The results of these experiments suggest strongly that the persistence of homologous cartilage transplants is due to the superb isolation of transplanted chondrocytes by the intercellular matrix. The only other tenable explanation for our results at this time is the remote possibility that chondrocytes do not carry tissue-specificity antigens or that they are not susceptible to foreign tissue antibodies formed against other tissues from the same donor animal.

Acknowledgment

We express our appreciation to D. Gordon Sharpe and John Schwab of the Department of Bacteriology, University of North Carolina School of Medicine, for technical assistance in performing the fourth experiment.

References

1. Bacsich, P. & W. J. B. Riddell. 1945. Structure and nutrition of the cornea, cartilage, and Wharton's jelly. Nature. **155:** 271.
2. Bacsich, P. & G. M. Wyburn. 1947. The significance of the mucoprotein content on the survival of homografts of cartilage and cornea. Proc. Soc. Edinburgh. **62:** 321.

3. Craigmyle, M. B. L. 1955. Studies of cartilage autografts and homografts in the rabbit. Brit. J. Plastic Surg. **8:** 93.
4. Dupertius, S. M. 1941. Actual growth of young cartilage transplants in rabbits. Arch. Surg. **43:** 32.
5. Gibson, T., R. C. Curran & W. B. Davis. 1957. Survival of living homograft cartilage in man. Transplantation Bull. **4:** 105.
6. Loeb, L. 1926. Autotransplantation and homotransplantation of cartilage in the guinea pig. Am. J. Pathol. **2:** 111.
7. Morgan, J. F., H. J. Morton & R. C. Parker. 1950. Nutrition of animal cells in tissue culture. I. Initial studies on a synthetic medium. Proc. Soc. Exptl. Biol. Med. **73:** 1.
8. Peer, L. A. 1958. Sex chromatin study to determine the survival of cartilage homografts. Transplantation Bull. **22:** 404.
9. Peer, L. A. 1955. Transplantation of Tissues. Chap. 12. **1:** 101. Williams & Wilkins. Baltimore, Md.
10. Schatten, W. E., *et al.* 1958. Biological survival and growth of cartilage grafts. Plastic Reconstruc. Surg. **22:** 11.
11. Schatten, W. E. *et al.* *Ibid.*
12. Carius, L. 1860. Libigs Ann. **116:** 1.
13. Young, L. 1949. The measurement of radioactive sulfur in biological material. Biochemical J. **44:** 179.

Discussion of the Paper

Edward C. Franklin (*New York University, New York, N. Y.*): As an immunologist who has been working a little on cartilage recently but who has done no work in the field of transplantation immunity, I think I shall have to draw primarily on the results of other observers in some of my speculations.

Peacock admirably tries in his report to answer the main question in the field of homotransplantation of cartilage: namely, why do cartilage transplants survive for such a long period of time?

The two theories that he has mentioned are prevalent at the moment. One of these states that cartilage either contains a poor antigen or does not diffuse its antigens into the host. The other states that cartilage may be protected by the mucopolysaccharide ground substance against a host response.

As we look at the composition of cartilage, it is composed primarily of cells, a matrix of chondroitin sulfate and chondromucoid protein, and some collagen. None of these constituents, to our knowledge, has ever been shown to be a strong antigen. Thus chondroitin sulfate, for instance, in most people's hands is now accepted as nonantigenic. Collagen is a weak antigen, and although a number of people have been able to produce antibodies to the collagen, the cartilage itself appears to be a rather weak antigen.

When we look at cartilage as a whole, the results are somewhat more controversial. Peacock states that cartilage in his very extensively immunized animals does not appear to be antigenic. On the other hand, Craigmyle, doing somewhat similar experiments in another species, did show that subsequently transplanted skin grafts in such animals were accelerated in their rejection. He also studied the regional lymph node response of these animals and seemed to think that this response, while qualitatively less than the other types of transplant responses, was similar to that seen in the other responses, namely, hyperplasia of the lymphatic cells, so that it appears that if cartilage is poorly antigenic it still seems to evoke an antigenic-type response.

The other theory more currently in vogue is the one that Peacock has also mentioned: namely, that cartilage is protected by its mucopolysaccharide ground substance and lives in a type of Algire chamber.

The experiments that Peacock has described seem to demonstrate clearly that, if cartilage is indeed antigenic, it is protected by this ground substance.

It seems likely, in the light of results obtained in other fields, that it may be possible now to test this on a more experimental level. Thomas and his coworkers have recently been able to show that when animals—rabbits, in particular—are given papain or large doses of vitamin A, there results a selective removal of chondromucoid protein from the cartilage of the ear and other areas of cartilage. This is accompanied grossly by the floppy-eared rabbit that most of you have seen and, on a microscopic level, by the significant loss or almost complete loss of metachromasia of the cartilage matrix. This is accompanied by profuse liberation of chondroitin sulfate in both the blood and the urine of the animal, so that we now have on hand experimental methods to remove selectively some of the matrix from cartilage.

It would be expected, therefore, that animals so treated might reject more rapidly the cartilage transplant than a control animal that has not had such treatment. Such an experiment, indeed, has been reported recently by Craigmyle in very preliminary form. Craigmyle gave one dose of papain to the host and to the recipient animal and, in an attempt to prolong the action of papain, also gave cortisone to the recipient. He demonstrated no effect on the transplants in either the host or the recipient, showing that, under his experimental conditions at least, the papain seemed to have no effect.

This was a very preliminary report. It consisted essentially of histological and histochemical studies, and did not include S^{35} incorporation. It is possible that the cartilage matrix was not removed completely and likewise possible that use of vitamin A rather than papain (which can have a stronger and more prolonged effect in these experiments) may have given different results.

In summary, may I say that the time now seems to have come when isolation of cartilage constituents and study of their antigenicity, as well as our ability to modify selectively the milieu of the cartilage as it grows, may well give us some answers to the problems that have been perplexing this field since the middle of the last century.

HARRIS: May I ask Peacock what was the temperature in his chamber? I was interested in the question of controlling the temperature in a chamber that was sitting on top of a constantly working electric stirrer or magnetic mixer.

PEACOCK: The temperature never went above 37° C. Actually, the top of the magnetic stirrer becomes warm, but we never did let it reach more than 37° C. Of course, 37° C. is sufficiently high: bacterial growth soon takes place in the apparatus. We did run a few tests of this apparatus in a cold room, but it did not seem to make any difference. A very good stirring effect occurs here with both the magnetic stirrers and the bubbling, and yet we never did have a temperature above 37° C. with which to contend.

HOMOGRAFTING OF SYMPATHECTOMIZED SKIN IN HUMANS*

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Numerous studies reported in the literature have shown that the homograft-rejection phenomenon can be blocked partially or delayed in many ways. One such study by Conway *et al.*[1] suggested that the epidermis and its adnexal structure may increase the antigenic propensity of skin. Subcutaneous autoburial† of skin (of mice) was performed to render the skin devoid of adnexal structures. Subsequently, this skin was recovered to serve as a homoplastic transplant. In these experiments the observed immune response was neither as prompt nor as complete as in the control animals. An evaluation of these studies in man was deemed worthwhile.

Individuals who have been subjected to sympathectomy (thoracic and lumbar) exhibit a gradual atrophy of the adnexa (hair follicles and sweat glands) of the skin. In this investigation the skin to be homografted was obtained from the leg of a human volunteer who had undergone lumbar sympathectomy 6 months earlier. The donor area was prepared further by electrolysis so that all hair follicles were killed. Microscopic sections of skin taken from the proposed donor site revealed atrophy of the sudoriparous and sebaceous glands and a thinning of the keratin layer.

In the first series a strip of skin 2 cm. wide and 12 cm. in length was cut at 0.018 in. from the leg of the specially prepared patient. The skin was then divided into 4 grafts, each measuring 2 by 2 cm. in area. Four paraplegic volunteers were used as recipients for the skin homografts. The recipient areas were prepared by removing split-thickness grafts by sharp dissection. The prepared homografts of skin were spread on the dermal beds and held in place by sutures of No. 5-0 black silk. All the grafts were dressed with Adaptic nonadherent gauze over which ordinary fluff gauze was placed to form a tie-over bolus dressing. The bolus dressings were removed on the fourth postoperative day. Sutures were removed from all the grafts on the fifth postoperative day.

Subsequently, skin was again obtained from the same donor and prepared as described above to provide a second series of homografts. In this series the grafts were maintained in tissue culture for 7 days before homografting. It was hoped that the antigenicity of skin with atrophic adnexa might be altered further by adaptation procedures *in vitro*. Our routine laboratory technique for maintaining tissues *in vitro* was followed. The 6 grafts obtained were placed in a sterile Petri dish on folded gauze moistened with sterile saline, and were transferred to the laboratory. To ensure the sterility of the specimens each piece of skin was soaked in a 300-μ/cc. suspension of Mycostatin in

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† The subcutaneous insertion into an animal of a portion of its own skin.

Earle's balanced salt solution for 30 min. Then it was soaked for 1 hour in a solution of Earle's saline containing 2000 μ/cc. of penicillin G and 10 mg./cc. of streptomycin. Subsequently, each square of skin was placed, dermal side down, on the floor of a 100-cc. T flask. All the folds were carefully smoothed out of the skin, and the fluid medium was added. The skin then floated on the medium with the dermis in contact with fluid, as in the technique of Gillette and Findley.[2]

Eagle's medium was used with the addition of a commercial penicillin streptomycin solution for tissue culture and a 100 μ/cc. suspension of Mycostatin. The medium was supplemented, 10 per cent by volume, with serum collected from the chosen recipient of each graft, as in Gaillard's technique.[3] Fifty mg. of cortisone was added to each flask. After 3 days an additional 10 per cent aliquot of the recipient's serum was added to each flask (Gillette, unpublished data). After 1 week of maintenance *in vitro* at 37° C. the skin grafts were removed, placed in Petri dishes on gauze saturated with Earle's balanced salt solution, and returned to the operating room. The skin was then homografted by the technique already described. The 6 voluntary recipients were paraplegic patients. Postoperative photographs were taken of the grafted sites before the dressings were applied. The grafts were again photographed on the fourth postoperative day and each day thereafter until they had definitely sloughed.

Examinations of the sites of the grafts and of the serial photographs revealed that the survival time of the skin homografts was prolonged in only 1 instance. One homograft that had been maintained in tissue culture survived for 40 days. Rejection of the 5 other grafts similarly treated and of the 4 grafts directly applied to the recipients occurred from 8 to 15 days after grafting. These results are comparable to those that would be expected in homografts of normal human skin. It is apparent, therefore, that elimination of the adnexal organs from the skin does not prolong its survival as a homograft in man. Furthermore, the conclusion may be drawn that the adnexal structures of the skin with their secretory products are not per se important factors in the homograft rejection phenomenon.

References

1. CONWAY, H., A. P. JEROME, R. B. STARK & D. JOSLIN. 1953. Observations on the development of circulation in skin grafts. VIII. The effect of burying whole-thickness grafts of skin prior to their homotransplantation. Plastic and Reconstructive Surg. **12:** 102.
2. GILLETTE, R. & A. FINDLEY. 1958. A simple technique for studying the effects of cultivation *in vitro* on skin grafts. Transplantation Bull. **5:** 124–125.
3. GAILLARD, P. J. 1950. Transplantation of cultivated parathyroid gland tissue in man. Ciba Foundation Symposium: Preservation and Transplantation of Normal Tissues. : 100. G. E. W. Wolstenholme and M. Cameron, Eds. Little, Brown. Boston, Mass.

AN INVESTIGATION OF HODGKIN'S DISEASE WITH RESPECT TO THE PROBLEM OF HOMOTRANSPLANTATION*

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Homograft rejection is believed to be an immune phenomenon that in its general details most closely resembles that group of immune reactions commonly termed delayed hypersensitivity or allergy of the bacterial variety.[1] Our interest in Hodgkin's disease was aroused by the reports of Schier and his co-workers[2,3] that patients with Hodgkin's disease are deficient in their ability to manifest this type of immune response. As a first step, experiments were carried out to attempt to confirm Schier's observations; this work has been reported in detail elsewhere.[4,5] Skin testing was carried out in patients with histologically proved Hodgkin's disease and in hospitalized controls, employing a standard battery of 7 antigens prepared from commonly encountered microorganisms to which the majority of individuals would be expected to react. The following materials were used: (1) dipththeria toxoid (Schick control solution), (2) streptokinase-streptodornase (SK-SD), (3) mumps skin-testing antigen, (4) mumps control solution, (5) *Trichophyton gypseum* extract, (6) *Candida albicans* extract, and (7) purified protein derivative (PPD) of tubercle bacilli, intermediate strength. Testing was carried out in the conventional manner, injecting 0.1 ml. of suitable dilutions intradermally and noting the immediate reaction, if any, as well as the response at 24 and 48 hours. A positive response was recorded if indurtion was present and if the reaction exceeded 5 mm. in diameter. To date 43 patients with Hodgkin's disease have been tested; all but 1 of these patients were ambulatory at the time of testing. None were receiving steroids or chemotherapy, although many were receiving regional X-ray therapy. The controls consisted of 208 hospitalized patients, excluding all individuals with malignancies or acute illness, as well as persons receiving treatment with steroids. The result of this testing is shown in TABLES 1 and 2. It is apparent that patients with Hodgkin's disease manifest a high incidence of nonreactivity or anergy in contrast to the controls. Our observations therefore confirm those reported by Schier and his co-workers. It then seemed logical to test the possibility that patients in whom delayed hypersensitivity is deficient might be anticipated to show tolerance or at least altered behavior to skin homografts, since homograft rejection seems to resemble delayed hypersensitivity in respect to many details of the immune process. Accordingly, permission was obtained from a group of 17 patients with Hodgkin's disease to carry out the placement of small skin homografts. These patients were all in fair to good general condition at the time of grafting

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and during the early period after it. None was receiving therapy with steroids or chemotherapeutic agents, although some of them had received such therapy in the past, and at the time of grafting some were receiving irradiation therapy in areas remote from the operative site. The grafts were full-thickness pieces of skin 1.5 by 2.0 cm. in dimension, tattooed with India ink in a regular pattern of evenly spaced dots to aid in subsequent follow-up. The behavior of the grafts was observed by careful inspection and palpation, together with photographic recording at frequent intervals for as long a period of time as possible. The results of this study are summarized in TABLES 3 and 4. Good initial take of the grafts was observed in all instances when the dressings were first removed at 7 to 10 days; there was minimal loss of portions of the graft in only a few instances. The subsequent behavior of the grafts appeared to fall into 3 broad categories, as follows: (1) normal early rejection at about 2 to 3 weeks with eventual gross sloughing of the graft, followed by healing of the graft site without evidence of residual tattooing; (2) complete healing of the graft without subsequent reaction and with indefinite survival of the graft unchanged save for minimal or moderate contraction in the manner of an autograft; and (3) initial take followed by the appearance of a delayed reaction usually beginning about 3 to 4 weeks after grafting and continuing for several weeks to months, leading eventually to partial or complete loss of the graft. Typically, this reaction appeared as the development of exudation and crusting, which usually started at the periphery of the graft and sometimes

TABLE 1
FREQUENCY OF POSITIVE SKIN REACTIONS TO DELAYED ALLERGENS IN PATIENTS WITH HODGKIN'S DISEASE AND CONTROL PATIENTS

	Schick control	SK-SD	Mumps	Mumps control	*Trichophyton*	*Candida*	PPD
Hodgkin's disease patients (43)	5%	26%	26%	0%	21%	9%	12%
Non-Hodgkin's disease patients (208)	10%	66%	88%	3%	61%	57%	52%

TABLE 2
FREQUENCY DISTRIBUTION OF POSITIVE RESPONSES TO DELAYED ALLERGENS IN HODGKIN'S DISEASE PATIENTS AND CONTROLS

No. positive responses	Hodgkin's patients (43)	Non-Hodgkin's patients (208)
0	24 (55%)	3 (1.4%)
1	4	8
2	9	41
3	1	59
4	2	60
5	2	30
6	0*	5
7	0*	2

* Two patients were tested with only the 5 most active antigens.

TABLE 3

SKIN HOMOGRAFTS IN PATIENTS WITH HODGKIN'S DISEASE

Case	Age	General condition	No. positive skin reactions	Known duration of graft	Behavior of graft
1	38	Fair	0	7 months	No reaction save for contraction of graft.
2	23	Good	0	14½ months	No reaction except for slight contraction in size.
3	47	Good	0	6 months	No reaction. Still completely intact when last seen.
4	30	Poor	4	18 days	Initial take followed by complete necrosis with slough. Healing without evidence of residual tattooing.
5	27	Good	1	2 weeks	Initial take. Rapid development of necrosis beginning at 12 days. Slough with healing. No evidence of residual tattooing.
6	29	Good	0	3–4 weeks	Initial take. Sudden reaction beginning at 3 weeks, with complete necrosis at 4 weeks. Slough and healing without residual tattooing.
7	35	Good	1	11½ months	Initial take. Reaction beginning at 21 days resulting in partial loss of graft, no further reaction after 5 months.
8	30	Good	0	31 months	Initial take. Intact at 1 month. Partial loss when seen at 2 months. Reaction ceased at 4 months, with no subsequent change. Partial retention of graft.
9	33	Good	1	14 months	Initial take. Reaction beginning at 2 weeks until 8 weeks, quiescence several weeks, then reaction lasting 3 weeks. No further reaction with partial retention of graft.
10	35	Fair	0	2½ months	Initial take with minor loss of 1 corner. Reaction at 5 weeks with quiescence at 11 weeks, with part of graft remaining.
11	32	Good	2	8 months	Initial take with minor loss. Lost to follow-up between 1 and 8 months. Partial retention of graft when seen at 8 months.
12	34	Fair	1	4 months	Initial take. Reaction beginning at 2½ weeks with eventual gradual loss without gross slough, no recognizable graft after 4 months.
13	33	Fair	0	5 months	Initial take. Reaction beginning at 17 days. Part of graft still present at time of death 5 months later.
14	24	Good	2	2½ months	Initial take. Reaction beginning at 2 weeks. Portion of graft still present when last seen.
Children					
1	12	Good	0	3 months	Initial take. Sudden slough between 9 and 12 weeks after transplantation.
2	14	Excellent	0	3–4 months	Initial take. Slough of a gradual type beginning 3 months after transplantation.
3	12	Excellent	0	10–17 days	Initial take. Rapid slough typical of homograft rejection in a normal person.

extended to cover the entire graft. When this reaction was first observed it was thought that it represented a somewhat delayed slough. However, after the crusts eventually separated, it was unexpectedly seen that underneath was a healed epidermal surface that showed a variable proportion of the original tattooed pattern of India ink dots that was readily recognized, with its spacing and sharp, punctate character unchanged. In some instances the crusting covered only part of the graft, and scaling or desquamation was seen over the central portion. In a number of grafts the reaction was repeated, with residual portions of the tattooed pattern still present after the second reaction subsided. In 3 of the 10 grafts in which the delayed reaction occurred there was eventually loss of all recognizable graft, usually around 3 months after placement of the graft. In the remaining grafts, after a period of time ordinarily in the vicinity of 3 to 5 months after placement, the reaction ceased, with indefinite persistence of the fragment for periods as long as 2½ years. The graft placed on 1 of the 3 patients, in whom its subsequent behavior was that of an autograft, was obtained from a female donor. A biopsy of this graft 6 months after placement not only showed the nuclear sex chromatin pattern characteristic of the female in the epidermal cells, but also demonstrated the persistence of sebaceous glands. At least in this instance it would appear that the clinical impression of persistence of the graft without reaction indicative of even gradual or possibly imperceptible replacement by the host is strongly supported by the histological evidence obtained by skin biopsy at a time long after graft rejection or replacement could be expected to occur. It is of interest that all 3 of the grafts that behaved like autografts were associated with anergy to the standard battery of antigens used in skin testing. However, 5 of the 10 patients who exhibited the late reaction in the graft, with indefinite persistence of portions of it, showed at least 1 positive skin reaction to the battery of antigens. This occurrence might be explained by persistence of a previously strong reactivity to 1 of the allergens in the face of an inability to develop reactivity to a new antigen. This possibility was investigated in the manner described below.

Table 4
Behavior of Skin Homografts in Hodgkin's Disease

Type of response	No. observed/No. tested
Like autografts	3/17
Delayed and protracted partial or complete rejection	10/17
Normal homograft rejection	4/17

Attempts to Induce Delayed Hypersensitivity to Dipththeria Toxoid

Uhr *et al.*[6] described a technique that they reported to be successful in inducing delayed hypersensitivity to diphtheria toxoid in previously insensitive individuals by the intradermal injection of a specific precipitate of diphtheria toxin-antitoxin. Experiments were carried out with this material in patients with Hodgkin's disease, using hospitalized patients as controls. The results are recorded in TABLE 5. It may be seen that none of the patients with Hodgkin's disease were able to develop reactivity to diphtheria toxoid, in contrast to the majority of the controls tested, thereby tending to support the idea that Hodgkin's disease patients might be incapable of manifesting delayed allergy when presented with a new antigen, although still capable of exhibiting delayed allergy to an antigen to which they conceivably had developed a strong reactivity in the remote past. Four of the 9 Hodgkin's disease patients tested with the specific precipitate showed at least 1 positive response to the standard battery of skin-testing antigens.

TABLE 5

PRODUCTION OF DELAYED HYPERSENSITIVITY TO DIPHTHERIA TOXOID

	No. positive	No. tested
Hodgkin's disease	0	9
Controls	9	14

TABLE 6

IMMUNOLOGICAL EVALUATION OF PATIENTS WITH HODGKIN'S DISEASE

	No. present	No. tested
(1) Normal or increased serum gamma globulin	8	8
(2) Typhoid-paratyphoid agglutinin formation	7	7
(3) Mumps complement fixation antibody formation	4	4
(4) Isoagglutinin titers normal	5	5
(5) Polio neutralizing antibody formation	0	4

Evaluation of Conventional Serum Antibody Capabilities

A small number of patients with Hodgkin's disease were studied from the standpoint of conventional antibody responses. The methods employed have been described elsewhere.[7] These observations are recorded in TABLE 6. It would seem that the serum antibody system in this small number of patients is not remarkably different from normal.

Anergy, the lack of responsiveness to antigens to which one would normally expect a high incidence of reactivity, has been recognized in many situations associated with cachexia or severe acute illness. One might therefore wonder whether the anergy seen in Hodgkin's disease may not be explained on this basis. It is noteworthy that a considerable number of these patients appeared to be in good general condition at the time of the skin testing that revealed the anergy. Nevertheless, to acquire more information on this point it was felt desirable to carry out small surveys in respect to several other groups of patients in which one might find advancing illness and cachexia. The result of this study is summarized in TABLE 7. Patients were arbitrarily classified as in poor condition or in fair to good condition on such bases as strength, state of nutrition, presence or absence of high persistent fever, severe pain, anorexia, and state of well-being. Obviously, some of the groups shown in TABLE 7 contain too few cases to be of other than passing interest. It may be seen from the first 3 groups, however, that the occurrence of anergy is very common in Hodgkin's disease, even though the patient is in good general condition, while anergy is unusual in comparable patients suffering from carcinoma or leukemia. Anergy is common in all cases of poor general condition. These observations make it attractive to postulate that anergy in Hodgkin's disease may indeed be intimately associated with the primary illness and may not be merely a nonspecific by-product of the terminal phase of the disease.

Passive Transfer of Delayed Hypersensitivity

It has been demonstrated in both man[8] and animals[9] that reactivity to allergens producing delayed-type reactions may be passively transferred to other individuals who are initially nonreactors to the allergen by means of the injection of living donor leukocytes, but not by the transfer of serum. To investigate further the apparent inability of Hodgkin's disease patients to manifest delayed hypersensitivity, experiments have been initiated in which attempted transfer of delayed hypersensitivity is tested. To date experiments have been carried out in a total of 9 patients with Hodgkin's disease and in 21 controls. In 4 of the control patients the test was carried out simultaneously with one of the Hodgkin's disease patients, using equal aliquots of the donor cells. Donors and recipients were of the same blood type. All patients were tested initially for their delayed skin reactions to the standard battery of allergens. The tests were repeated 48 hours after injection of the cell suspension. All of the Hodgkin's disease patients tested were initially anergic to the standard battery of skin tests. Control patients were selected who failed to show a delayed reaction to at least 1 of the allergens to which the donor was highly sensitive. Donors consisted of healthy laboratory personnel who were previously skin-tested with the standard battery of allergens and whose skin sensitivity was therefore known. A unit of blood (500 ml.) was collected from the donor into a siliconized or plastic container. Rapid sedimentation and separation of the white-cell fraction were effected by the addition of 6 per cent dextran (Plavolex). The blood was allowed to settle in a siliconized separatory funnel, and the red cell layer was removed. The plasma containing the white

TABLE 7
INCIDENCE OF ANERGY TO DELAYED ALLERGENS IN VARIOUS DISEASES

	General condition	
	Good No. anergic/No. tested	Poor No. anergic/No. tested
Hodgkin's disease	19/36 (53%)	5/7 (71%)
Carcinoma	0/22	15/34 (44%)
Leukemia	1/18 (6%)	4/8 (50%)
Non-Hodgkin's lymphoma	2/9 (22%)	5/7 (71%)
Multiple myeloma	0/4	3/6 (50%)

blood cells was then gently centrifuged, the plasma poured off, and the packed white cells resuspended in balanced salt solution. The concentrated white cell fraction was then injected subcutaneously into the recipient individual. Where simultaneous control patients were used the white cell fraction was divided into equal portions, and one half was injected into the control patient and the other into the patient with Hodgkin's disease. Total nucleated cell counts were made on small aliquots of the white cell fraction, and estimates were made of cell viability, employing the technique of vital staining with trypan blue dye. Cell injections were always accomplished within 2 hours of the time of obtaining the blood specimen. In one instance the blood specimen was not fractionated, but simply divided into 2 portions that were given intravenously after cross matching. The total dose of cells administered ranged from approximately 0.5 to 3.5 billion nucleated cells and averaged about 1.0 to 1.5 billion cells in most instances. None of the patients tested were receiving therapy with steroids or chemotherapeutic agents, although several of the Hodgkin's patients were receiving regional irradiation therapy. The result of these preliminary experiments is summarized in TABLE 8. It is seen that in no instance was it possible to demonstrate the passive transfer of delayed hypersensitivity to a patient with Hodgkin's disease, although 20 of the 21 controls (including the 4 simultaneous controls) showed distinctly positive results. It is strongly suggested from this preliminary experience that patients with Hodgkin's disease are able to interfere with, or fail to manifest, delayed hypersensitivity even when it is passively transferred by way of white blood cell suspensions.

TABLE 8

PASSIVE TRANSFER OF DELAYED ALLERGY IN HODGKIN'S DISEASE AND CONTROLS

	No. successful	No. attempted
Hodgkin's disease	0	9
Controls	20*	21

* Including 4 simultaneous controls receiving the same dose of the same donor cells as did the Hodgkin's patients.

Comment

Hodgkin's disease, like congenital agammaglobulinemia, appears to represent a unique experiment of nature from which much can be learned about immune phenomena and their relationship to the process of homograft rejection. One is tempted to view Hodgkin's disease immunologically as the opposite side of the coin to agammaglobulinemias in that in the latter case serum antibody formation is markedly deficient or absent in the face of retention of delayed hypersensitivity,[10] while the former condition appears to possess the reverse combination. In both conditions the response to homografts of skin is markedly abnormal. Apart from the special case of identical twins, homografts of skin in humans regularly are rejected completely within 2 weeks of placement.[11] Certain factors have been reported to alter the normal rejection of skin homografts in man, such as marked similarity of blood types,[12] uremia,[13] and the presence of extensive burns.[14] The retention of skin homografts in Hodgkin's disease seems to represent a more pronounced deviation from the normal in respect to the slowness of reaction in most instances and the absence of any obvious reaction in others. It is evident that much further work will be necessary to establish the tentative interpretations of the immunological abnormality in Hodgkin's disease reported here. It is readily admitted that the observations reported may be susceptible to an interpretation other than that given. It is possible that gradual replacement of the skin homografts by the host tissues may take place without obvious graft reaction, although this seems unlikely in view of the persistence of India ink markings in a characteristic pattern of sharp dots. Possibly the epidermis may be lost from the graft with replacement by the host and with retention of only the dermal pad of the graft in those cases where late reactions occurred, but this remains to be determined. Whatever the mechanism, the reaction to skin homografts in patients with Hodgkin's disease is far different from that observed in normal individuals. What is of even more interest is the evidence that patients with Hodgkin's disease not only are deficient in their ability to respond with delayed reactivity to appropriate antigens, but possibly may even be able to suppress or interfere with the manifestation of passively transferred reactivity to antigens normally inducing delayed hypersensitivity. Since so little is known about the actual mechanism whereby delayed hypersensitivity is passively transmitted, one cannot go further at the present time.

Summary

Patients with Hodgkin's disease show a high incidence of anergy (nonreactivity) when skin-tested with solutions of antigens prepared from commonly encountered microorganisms to which the vast majority of control patients react. The reaction to skin homografts in Hodgkin's disease is abnormal, with delayed prolonged incomplete reactions being common. In some instances a complete absence of reaction to the homograft was observed. In a preliminary analysis of the deficit in delayed hypersensitivity in Hodgkin's disease it was found that attempts to induce hypersensitivity to a new antigen were unsuccessful, as were attempts to transfer delayed hypersensitivity passively by the injection of living white blood cells, although such attempts were successful in control patients. It is tentatively suggested that Hodgkin's disease represents a condition in which immune reactions of the delayed-hypersensitivity variety are primarily suppressed at an early stage. Associated with this is a markedly impaired ability to reject homografts of skin.

References

1. Lawrence, H. S. 1957. Similarities between homograft rejection and tuberculin-type allergy; a review of recent experimental findings. Ann. N. Y. Acad. Sci. **64**(5): 826.
2. Schier, W. W. 1954. Cutaneous anergy and Hodgkin's disease. New Engl. J. Med. **250:** 353.
3. Schier, W. W., A. Roth, G. Ostroff & M. A. Schrift. 1956. Hodgkin's disease and immunity. Am. J. Med. **20:** 94.
4. Kelly, W. D., R. A. Good & R. L. Varco. 1958. Anergy and skin homograft survival in Hodgkin's disease. Surg. Gynecol. Obstet. **107:** 565.
5. Kelly, W. D., R. A. Good, R. L. Varco & M. Levitt. 1959. The altered response to skin homografts and to delayed allergens in Hodgkin's disease. Surg. Forum. **9:** 785.

6. Uhr, J. W., S. B. Salvin & A. M. Pappenheimer, Jr. 1957. Production of delayed sensitivity to protein without detectable circulating antibody. Ann. N. Y. Acad. Sci. **64**(5): 877.
7. Good, R. A. & S. J. Zak. 1956. Disturbances in gamma globulin synthesis as experiments of nature. Pediatrics. **18:** 109.
8. Lawrence, H. S. 1949. The cellular transfer of cutaneous hypersensitivity to tuberculin in man. Proc. Soc. Exptl. Biol. Med. **71:** 516.
9. Chase, M. W. 1945. The cellular transfer of cutaneous hypersensitivity to tuberculin. Proc. Soc. Exptl. Biol. Med. **59:** 134.
10. Good, R. A., R. L. Varco, J. B. Aust & S. J. Zak. 1957. Transplantation studies in patients with agammaglobulinemia. Ann. N. Y. Acad. Sci. **64**(5): 882.
11. Rogers, B. O. 1957. The genetics of skin homotransplantation in the human. Ann. N. Y. Acad. Sci. **64**(5): 741.
12. Woodruff, M. F. A. & T. M. Allan. 1952–1953. Blood groups and the homograft problem. Brit. J. Plastic Surg. **5:** 238.
13. Dammin, G. J., N. P. Couch & J. E. Murray. 1957. Prolonged survival of skin homografts in uremic patients. Ann. N. Y. Acad. Sci. **64**(5): 967.
14. Kay, G. D. 1957. Prolonged survival of a skin homograft in a patient with very extensive burns. Ann. N. Y. Acad. Sci. **64**(5): 767.

Discussion of the Paper

Chase: Is there any parameter of observation of which you are aware, in respect to which these four individuals stood apart from the thirteen others?

William D. Kelly: Two of these patients (in terms of their delayed hypersensitivity) showed positive skin reactions; two of them showed negative skin reactions to the standard battery of antigens that I showed.

Chase: So they were not completely anergic?

Kelly: That's correct.

Chase: I see. Have you tested patients with sarcoidosis who also, of course, may remain in excellent health?

Kelly: No, sir; we have not. We have skin-tested a small group of these patients, and have not found a high incidence of anergy, but we have only a very few of these patients.

John P. Merrill: I make one comment before presenting the antithesis of what the present speaker just discussed, and that is that this problem about the anergy in Hodgkin's disease illustrates a very important point in which we have been interested. Man as an experimental subject for the study of transplantation immunity is quite a different proposition from the inbred mouse. Both have varying degrees of antigenicity, of course, and they have varying degrees of ability to produce antibodies, both of which are probably genetically determined.

However, man also has chronic illnesses, and we have already stressed the point that such incidents as burns, uremia, and trauma may modify this.

I suggest that perhaps we should consider the effect of chronic disease in our own animal colonies. Perhaps some of the discrepancies in the results in animal colonies, at least in ours, are attributable to the fact that not all our animals are continuously in perfect condition.

I think it is also important to stress a fact already mentioned, that Hodgkin's is not a single disease. It is a variety of diseases of the lymphopoietic system, varying cell types, varying degrees of anaplasia, and varying degrees of sensitivity to radiomimetic agents. Therefore, the response may not be that of Hodgkin's disease, but of the degree and extent of the involvement of the lymphopoietic system. I think that possibly this is shown by the results here, which varied all the way from autograftlike results to perfectly normal rejections.

I ask whether at any time therapy made any difference, whether electrophoretic patterns were done on these individuals.

We have had an opportunity to study another disease in man that is sharply contrasted with Hodgkin's in the sense that these individuals apparently produce antibodies, if you will, to their own tissues.

I am not suggesting that the antibodies they produce necessarily cause clinical phenomena in this disease. In fact, there is good evidence that the antibody, the globulin isolated, is perhaps not closely related in this regard, but the two phenomena do correlate. I am talking about disseminated lupus erythematosus.

In these individuals, at least, the serum antibody is directed against the DNA, and I think this is of interest in consideration of what we think may be the transplant antigen. The antibody is directed against their own DNA and against homologous and even heterologous DNA. There is another relationship to transplantation immunity in that these individuals do show that perivascular cell infiltration, vascular disease, and their disease is capable of being modified by cortisone, whole-body irradiation, and 6-mercaptopurine, which also are known to effect transplantation immunity to some degree.

One might expect, if this were true, that such individuals might show a delayed type of hypersensitivity to the sort of antigen against which the serum antibody is directed and, indeed, this sort of thing has proved to be true. It is of particular interest, I think, that this delayed type of hypersensitivity is directed against their white cells.

CHASE: There is, then, no question regarding the fact that the Hodgkin's cases present a different situation, regardless of the kind of graft applied?

GUSTAVE J. DAMMIN: May I ask whether, when using female grafts on male recipients, there was any influence of sex difference on the duration of survival of the grafts? Also, did blood group differences play an essential role in the duration of survival?

One photomicrograph showed some cell infiltration. I think this is a most important point because, when we compare this with what happens in chronic uremia, we find that the uremic patient has no difficulty in elaborating plasma cells, and such cells appear in large numbers in skin grafts and in the kidney homograft, but the subsequent rejection may be delayed or may not even be perceptible for long periods.

I wonder whether the cells that are elaborated perhaps in Hodgkin's and also in the chronic uremic instances are not as effective as those in a normal recipient; or perhaps the tissues are not as reactive to what the cells may be releasing. One also wonders whether, when rejection enters a prolonged phase, there may not be something enhancing that is elaborated that makes these cells ineffective in a situation in which you might expect them to play some role in rejection.

I now propose to describe briefly what the correlations are among cellular infiltration of the dermis, the elastic fiber pattern, and the epidermis in skin graft rejection.

One may see cells around vessels and a very slightly altered epidermis, but a remarkably abnormal elastic fiber pattern.

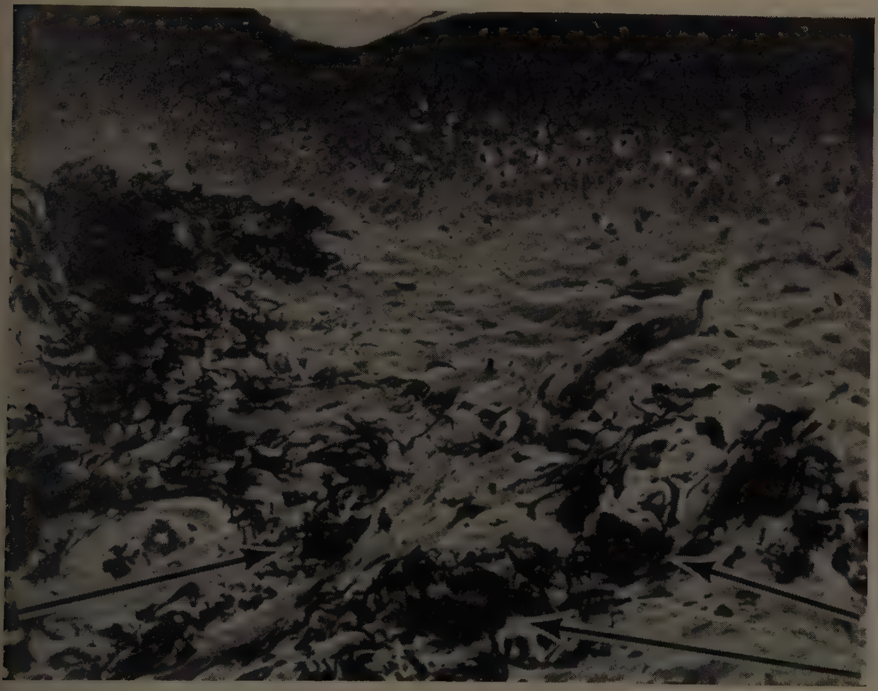

FIGURE 1. Biopsy of tattooed skin homograft. This was taken 2½ years after placement of the graft upon a recipient who had sustained extensive burns. The orientation of the cells in the epidermis is normal. The upper dermis in the right half of the field is completely devoid of elastic fibers, and those in the left half present a distorted pattern. This alteration accompanies loss of the graft epidermis, and that shown here has grown from the recipient epidermis. Evidence for persistence of other components of the dermis is shown by the masses of tattoo pigment in the lower half of the field, indicated by arrows. Reproduced by permission of *Transplantation Bulletin* (**6**: 429, 1959).

Instead of having a delicate pattern that shows projections up to the base layer of the epidermis, only fragments may remain, and this we have found a very good hallmark of rejection, that is, a distortion with resorption of the elastic fibers in the upper dermis.

It appears that resorption of elastic fibers may occur before there is incontrovertible microscopic evidence of rejection of the epidermis, and that the first phase in the rejection is the presence of cells, and this is followed very soon by resorption of the elastic fibers.

In connection with Kelly's observation that tattoo pigment will persist for long periods, I now mention a biopsy of a tattooed homograft that was put on a burn recipient 2½ years before. This biopsy (FIGURE 1) shows the distorted elastic fiber pattern that can be correlated with a loss of graft epidermis. However, there is a persistence of tattoo pigment indicating further that some elements of the dermis have survived, but that the epidermis has been replaced by growth from the recipient.

KELLY: Merrill has asked whether there were any differences dependent upon the therapy these patients were receiving. Frankly, I could not see that there were any differences, although I do not think we have enough observations to draw any firm conclusions.

However, at least one of the patients who rejected the homograft early, in a typical manner, was a severely ill patient who had received innumerable courses of nitrogen mustard and irradiation for a considerable period in the past before we tested him, yet he rejected his homograft in an apparently normal manner.

On the other hand, several of the patients who had apparent indefinite survivals of their homografts were in excellent condition and had received only one course of chemotherapy some weeks prior to our application of the homograft; they were not receiving any therapy during the time of the homograft's application or survival in subsequent weeks.

We have studied electrophoretic patterns in these patients and we have seen no gross abnormality, although this has not been studied in great detail.

Green has described his experiences with Hodgkin's disease. I read his paper in *Blood*. He had only four patients, I believe, that had Hodgkin's disease. I suggest, first, that this may not be an adequate sample on which to base an opinion, particularly in view of the type of variations that we have seen in our own experience.

Second, I do not think that he had any direct technique for identifying persistence of the graft dermis; therefore, he may have been led to interpret these "delayed rejections" around 30 days as being typical rejections, which we do not interpret in the same manner.

The percentage of sex markers present is about 25 per cent in one skin biopsy. I agree that gradual replacement may occur, and it very likely does in many of these grafts that showed this so-called delayed reaction, a term we prefer to use instead of considering it a typical, normal reaction.

Dammin asked about sex differences. We have not had a sufficient number of patients to evaluate this. All but three of our patients were males and most of them received male skin homografts except for two or three patients who received female skin. Two of these, unfortunately, fell in the group of early rejections and we had only one patient who had a long survival; therefore, we cannot evaluate this inadequate number of patients as yet.

We made no effort to determine general blood groups in these transplantations. Accordingly, I have nothing to say about that aspect.

Finally, I do not wish to leave the impression that we claim that these grafts are surviving indefinitely without the possibility of gradual replacement. I think that this replacement is a very definite possibility. Our principal contention is that the rejection pattern is definitely abnormal and that, grossly, some of these grafts appear to survive indefinitely, at least during our period of inspection.

IRA GREEN (*Hematology Department, Montefiore Hospital, New York, N. Y.*): In a study published elsewhere[1] it was shown that homograft survival in patients with lymphomas was only modestly prolonged: the longest homograft survival in a patient with Hodgkin's disease was 34 days. Another patient, terminally ill with reticulin cell sarcoma, died with his graft intact at 79 days. These results differ from those recorded by Kelly *et al.*[2] The difference, I believe, is due to the amount of collagen included in the homograft. This is illustrated in FIGURE 1. We used split-thickness skin, 0.0010 to 0.0014 inches thick, obtained with a dermatone. Kelly *et al.* used full-thickness skin grafts containing a large amount of collagen. When the epidermis dies, this thick collagen pad may survive and remain dry. Gradual overgrowth and interdigitation of host epithelium then occurs.[3] The inert survival of collagen and connective tissue is well illustrated in the use of dead aortic homografts. The use of small amounts of collagen in our skin homografts made the dry dermal pad phenomenon less likely.

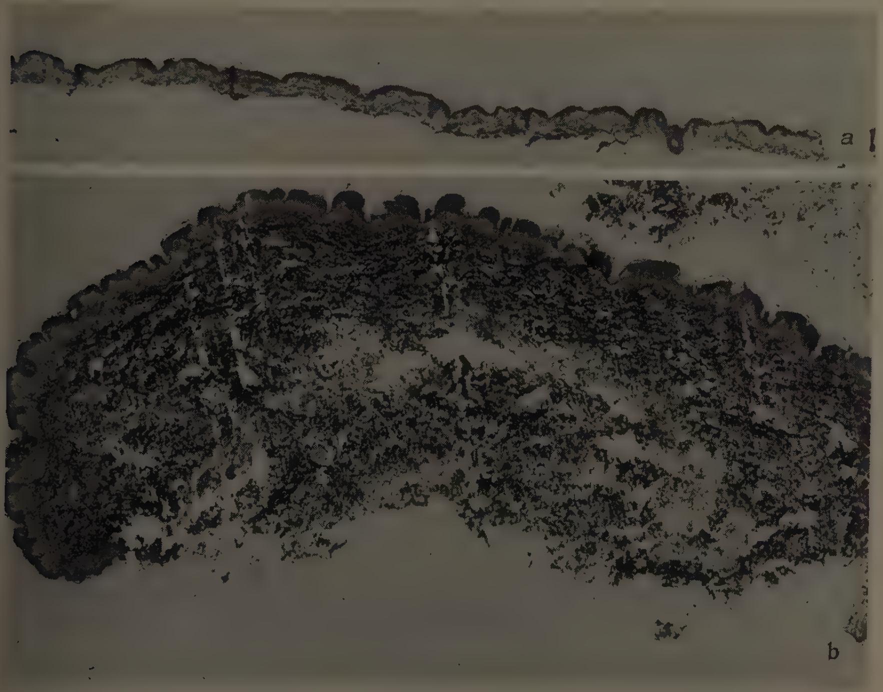

FIGURE 1. (*a*) Split-thickness graft of the type used in these experiments (0.0014 in.) ×25. (*b*) Full-thickness graft, showing large amount of collagen. ×25.

I feel that the best criterion for survival would be the demonstration of female chromatin material in a female skin graft placed on a male patient. However, the interpretation of this technique also may be difficult. Woodruff and Lennox[4] cross-grafted a human pair (of differing sex) of blood chimeras and found the grafts grossly survived. The female twin showed survival of the male skin graft based on chromatin marker techniques. The male twin, on the other hand, showed a chromatin pattern indicating replacement of the female skin. Woodruff and Lennox feel that this result may not rest on an immunological basis and suggest that a similar type of replacement might occur also in autografts.

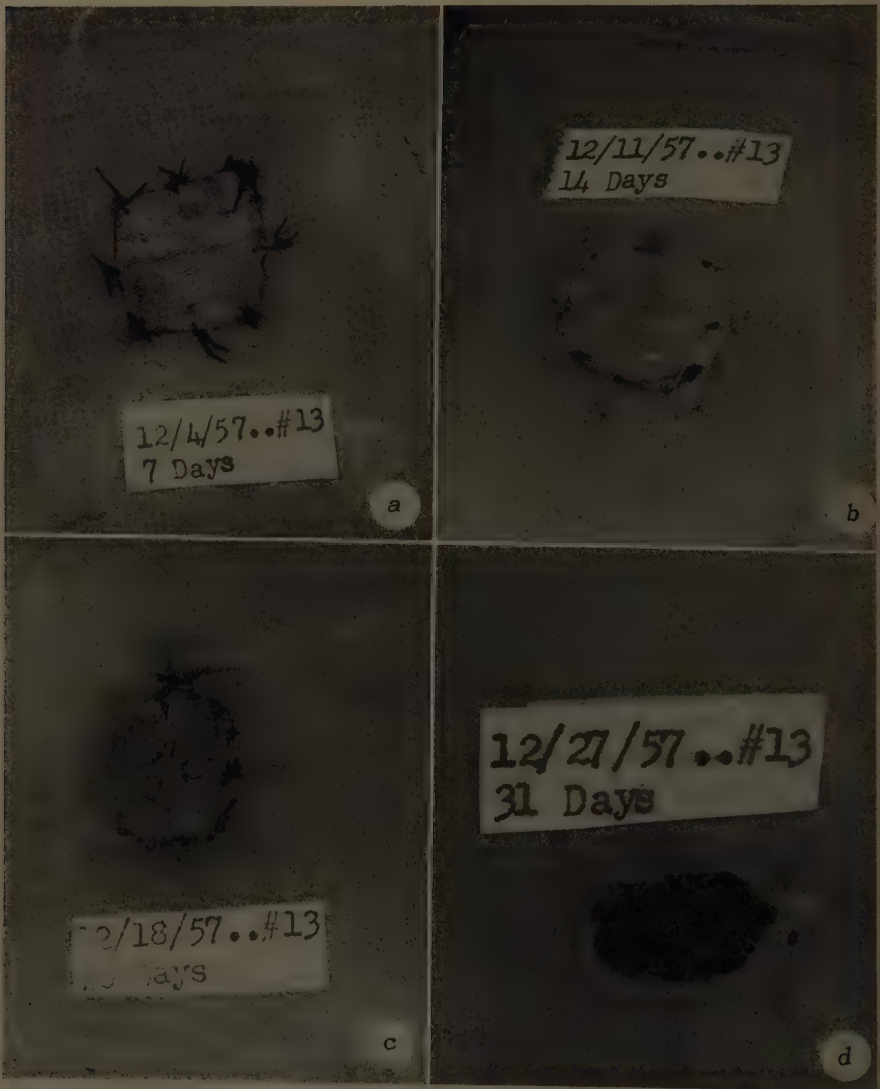

FIGURE 2. Stages in the rejection of a homograft. (*a*) Take of graft at 7 days. (*b*) Graft intact at 14 days. (*c*) Graft becomes dark red and is wrinkled. (*d*) Graft becomes converted into a mass of necrotic debris.

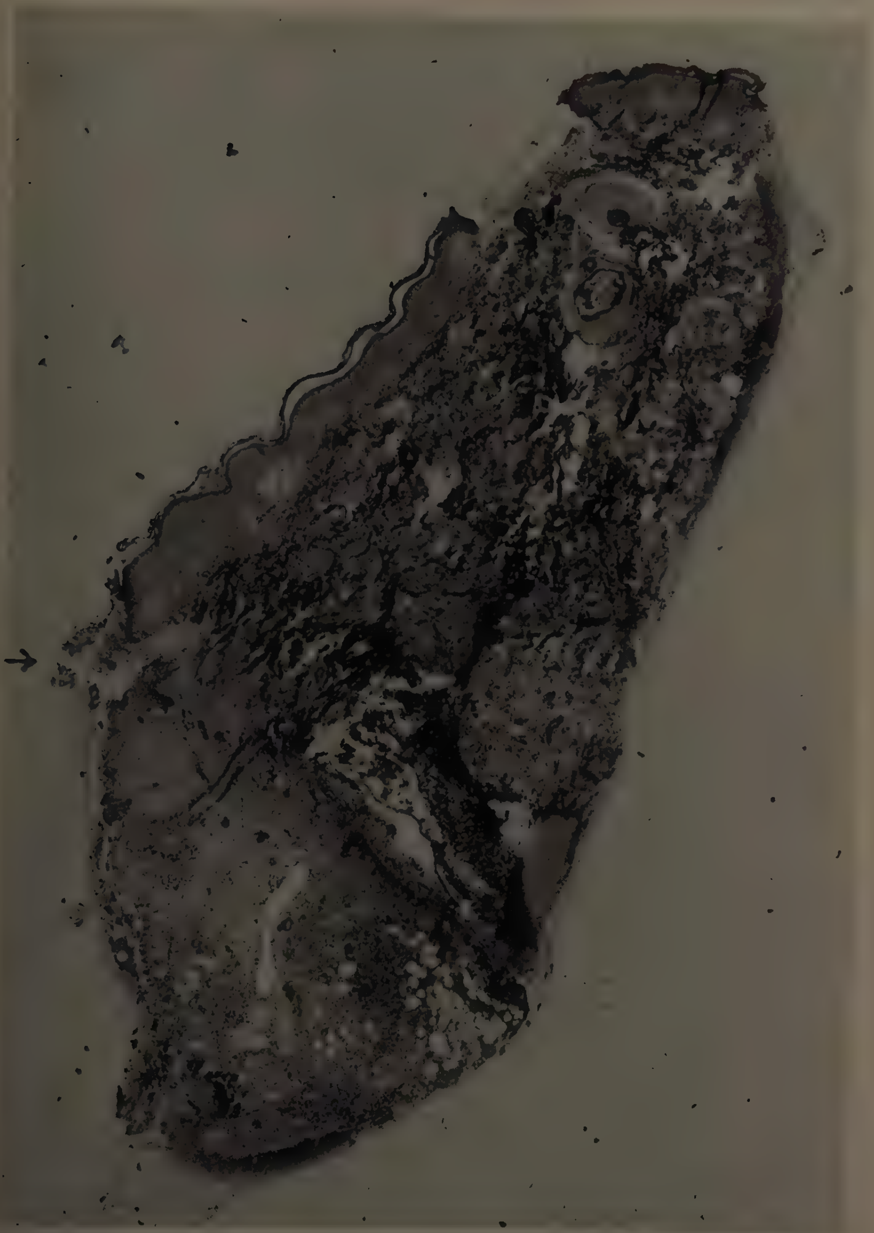

FIGURE 3. Low-power view of homograft in process of rejection. (*Below*) Loose connective tissue infiltrated with lymphocytes. The epidermis has disintegrated. (*Above*) The host side of the homograft, showing thick, normal host collagen, against which is host epidermis. Arrow shows junction. ×40.

In a preliminary study[5] it has been shown that maternal homografts may survive for longer periods of time in patients with Hodgkin's disease.

It is apparent that many more cases of lymphomas, as well as patients with other chronic illness, must be studied before a final answer to the question of homograft survival in lymphomatous states is forthcoming.

I suggest the use of thin split-thickness homografts and female chromatin-marker techniques for most accurate appraisal of the results.

FIGURES 2*a* and *d* show the rejection of a 0.0014-thick homograft in a 27-year-old patient with Hodgkin's disease (stage III). This patient had previously received extensive radiotherapy. This case is patient No. 6 of our previously reported series.[1] The survival of this graft was judged to be 27 days.

FIGURE 3 shows a low-power view of a homograft being rejected (14 days) in a patient with Hodgkin's disease (case No. 7[1]).

Summary. In my experience homograft rejection in patients with lymphomas is only modestly impaired, except when the patient is terminal and in certain instances where maternal homografts are used.

References

1. GREEN, I. & P. F. CORSO. 1959. A study of skin homografting in patients with lymphomas. Blood. **14:** 235.
2. KELLY, W. D., R. GOOD & R. VARCO. 1958. Anergy and skin homograft survival in Hodgkin's disease. Surg. Gynecol. Obstet. **107:** 565.
3. ROGERS, B. O. 1951. Guide and bibliography for research in the skin homograft problem. Plastic Reconstruc. Surg. **7:** 169.
4. WOODRUFF, M. F. A. & B. LENNOX. 1959. Reciprocal skin grafts in a pair of twins showing blood chimaerism. Lancet. **2:** 476.
5. GREEN, I., M. INKELAS & L. ALLEN. 1960. Hodgkin's disease—a maternal to fetal lymphocyte chimaera? Lancet. **1:** 30.

ALTERATION OF THE HOMOGRAFT RESPONSE BY ANTIMETABOLITES*

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Introduction

Schwartz and associates[1-3] have published reports demonstrating the suppression of humoral antibody formation to simple protein antigen by 6-mercaptopurine (6-MP) in the rabbit. These papers raised the interesting possibility that 6-MP and its related compounds also might suppress the homograft reaction. The most exciting possibility was that a drug-induced state of immunological tolerance to skin homografts might result from 6-MP treatment of the host, as these authors have been able to induce such a state of nonreactivity to protein antigen by treating rabbits with relatively high doses at the time antigen is presented. The work of Gorer[4] on the H-2 system of antigens in homograft reactions in mice, the demonstration by Good and his associates[5] of a diminished to absent skin homograft response in some patients with agammaglobulinemia, and the production of specific skin homograft rejection by local injection of immune serum by Stetson and Demopoulos[6] have provided evidence that humoral antibody may play a role in the rejection of homografts. There is thus reason to expect that 6-MP might prolong the survival of homografts through a mechanism of suppression of humoral antibody formation. Since one of the chief effects of 6-MP in high doses in mammals is the production of bone marrow hypoplasia,[7] it also might be expected to counteract the cellular aspects of the homograft reaction implicated by the millipore chamber experiments of Algire,[8] the demonstration by Mitchison[9] that transplantation immunity to tumor transplants can be transferred passively by cells, the work of Billingham *et al.*,[10] who demonstrated the passive transfer of transplantation immunity for normal tissues by cells, and the studies of Brent[11] indicating that cells of an animal rejecting a homograft will produce an intense delayed reaction when injected intradermally into the donor.

We have reported recently that 6-MP in high doses does, in fact, exert a suppressive effect on the skin homograft response in rabbits as long as treatment is maintained.[12] The present report is concerned with a further evaluation of the 6-MP effect on skin homograft survival in rabbits and mice with regard to dose levels, the time at which treatment is instituted, and the effect on second-set skin homografts. A brief account of our experience with other analogues of naturally occurring purines and pyrimidines also will be given.

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Materials and Methods

In all experiments reciprocal full-thickness cross grafts of skin were exchanged between abdominal sites in albino New Zealand strain rabbits weighing between 2.5 and 3.0 kg. and black and white Dutch strain rabbits weighing 1.0 to 2.5 kg., randomly distributed as to sex, that were obtained from commercial sources and maintained on Purina Rabbit Chow *ad libitum*. Both strains were equally distributed between treated and nontreated groups. The techniques of skin grafting have been described in previous communications from this laboratory.[13,14] Each animal, however, received a homograft and an autograft, and those whose autografts did not take are excluded from the analysis. Take of the autograft, however, occurred in almost all of the animals. No antibiotics were administered. Groups usually averaged 10 treated animals and 10 untreated animals. The 6-MP was supplied as an amorphous yellow powder prepared fresh at weekly intervals by dissolving preweighed aliquots in 0.1 N sodium hydroxide in a ratio of 1 mole of 6-MP to 1 mole of alkali, and the resulting solution diluted with normal saline to a final concentration of 15.2 mg./cc. All injections of the drug were administered via the ear veins.

In the early experiments serial observations were made of weight at 4- or 5-day intervals, of peripheral leukocyte counts, and of hemoglobin, and have been previously reported.[12] Briefly, it was noted that leukocyte counts and hemoglobin levels fell profoundly after the third week of treatment at dose levels of 12 mg./kg./day or higher, but did not occur with lower dosages.

The condition of skin grafts was assessed by daily gross observation. The end point of homograft rejection was usually clear-cut in the rabbits. On occasion, when there was doubt in the observer's mind as to graft viability the capillary circulation in the graft was observed, using a Leitz dissecting microscope according to the method outlined by Taylor and Lehrfeld.[15] Microscopic sections stained with hematoxylin and eosin and with methyl green-pyronin stains were also made of all apparently viable homografts and autografts in animals that died during the course of treatment. In general, a paucity of cellular response to skin homografts in treated animals as compared to controls was noted, as reported previously. Autopsies were performed on these animals in an effort to ascertain the cause of death. In all animals so examined no outstanding lesions other than hypocellularity of the bone marrow were noted, although most had a profuse nonbloody diarrhea prior to death. It has been noted that 6-MP in fatal doses produces denudation of the intestinal epithelium and degenerative changes in the liver.[16] No such instances of these lesions have been noted in our rabbits.

Results

Treatment with 6-MP initiated at the time of, or several days prior to, grafting. In this series of experiments 4 dose schedules were compared. Group I consisted of animals treated at a dose level of 6 mg./kg./day until slough of the homograft occurred. Group II consisted of animals treated with 12 mg./kg./day until slough of the homograft occurred. Group III consisted of animals treated with 12 mg./kg./day for 14 days. Group IV consisted of animals treated with 25 mg./kg. for 3 days prior to grafting and 12 mg./kg. for 9 days after grafting. Groups V and VI comprise untreated animals. The data are summarized in TABLE 1.

These data demonstrate that 6-MP in all dosage schedules used produced a prolongation of homograft survival. The prolongation was greatest in animals receiving 12 mg./kg./day. It is of interest to note that in the group in which treatment was discontinued after 14 days, which was the mean slough time for controls, the grafts continued to survive an average of 10.4 days. This period approximates the mean slough time of controls and suggests that the homograft response was suppressed during the period of drug treatment and made its appearance following cessation of treatment. It should be pointed out that the data presented in TABLE 1 concern only those animals whose survival is of sufficient duration for graft slough to occur. In all treatment groups there were animals that died with viable homografts as early as 5 days and as late as 27 days. Pretreatment prior to grafting with high doses had little beneficial effect on graft survival and appeared to increase the toxic manifestation of the drug. The dosage effect is clearly illustrated in FIGURE 1.

Table 1

Treatment Starting at or Before Grafting

Group no.	Treatment	Mean slough time (days)	Range (days)	S.E. of mean (days)	No.
I	6 mg./kg./day	18.4	13–27	1.1	7
II	12 mg./kg./day	23.3	19–28	1.1	7
III	12 mg./kg./day for 14 days	24.3	23–26	0.4	10
IV	25 mg./kg./day for 3 days before grafting 12 mg./kg. for 9 days after grafting	20.7	15–25	1.3	6
V	Control	14.7	11–16	0.4	9
VI		14.1	13–17	0.6	8

Mean difference between the experimental and control groups is statistically significant (P < 0.01).

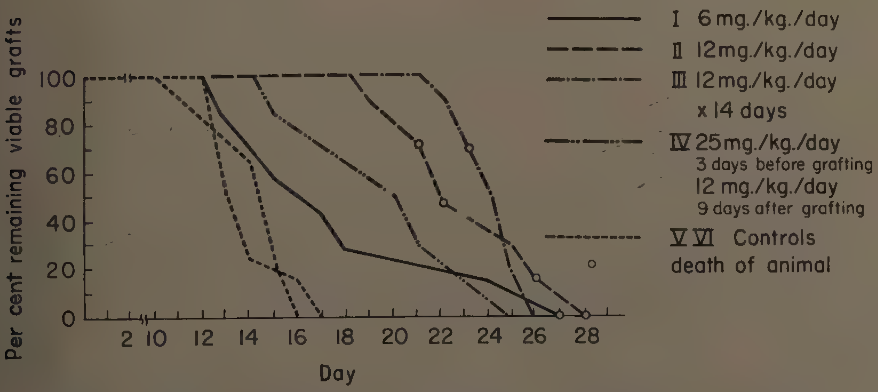

Figure 1. Effect of continuous treatment initiated at or immediately before grafting.

Eight surviving treated animals from Group III had second-set homografts exchanged between them 2 weeks after slough of the primary graft. Six controls from the same experiment had second-set homografts 2 weeks after slough of the primary graft. No treatment was given to either group. The survival of second-set skin homografts in animals receiving treatment during primary grafting was 10.6 days (range 10 to 12), compared to a mean survival time of 10.2 days (range 9 to 11) in the controls. It thus appears that 6-MP treatment during primary grafting has no effect on the subsequent development of a second-set response.

Effect of treatment begun and concluded before grafting. An experiment was designed to evaluate the effect of pretreatment alone on the survival of skin homografts placed on the animals the day after treatment was stopped. Peripheral leukocyte counts were done on all treated animals at weekly intervals. Treatment and control groups were set up as follows:

In Group I, 10 rabbits were pretreated with a dose of 12 mg./kg./day for 14 days prior to grafting; in Group II, 11 rabbits were pretreated with 10 mg./kg./day for 2 weeks prior to grafting; in Group III, 8 rabbits were pretreated with 10 mg./kg./day for 2 weeks prior to grafting; in Group IV, 10 animals received no treatment and served as controls. The results are summarized in TABLE 2.

TABLE 2
EFFECT OF PRETREATMENT ON THE SURVIVAL OF SKIN HOMOGRAFTS

Group no.	Dose	Mean slough time	Range	Number surviving to grafting	Per cent mortality
I	12	13.3	14	3	75
II	10		15–20	3	70
III	10		7–14	3	70
IV	Control	14.5	9–18	10	0

These data do not indicate any significant effect of pretreatment on the survival of skin homografts. A striking mortality due to treatment is noted. The deaths occurred during treatment in every instance except for 1 animal who died after grafting. This mortality was not due to profound bone marrow suppressions, as all treated animals had peripheral leukocyte counts at weekly intervals, the lowest count being 3850 among the animals dying. This high mortality contrasts with low mortality (1 of 10 animals) in the group of animals receiving 12 mg./kg./day for 14 days after grafting. Group I and Group IV animals received 20 mg./kg. of bovine plasma albumin* 14 days prior to grafting, all animals were bled on the day of grafting and the sera were tested for precipitating antibody by a capillary tube technique (Condie and Good, unpublished data). None of the 3 treated animals had detectable antibody to bovine plasma albumin, while all but one of the 10 controls formed antibody.

Trentin[17] demonstrated that X-irradiation followed by an infusion of homologous marrow produced a state of chimerism in mice, and Wilson *et al.*[18] have demonstrated the same thing in rabbits. In an effort to prevent our animals from dying during pretreatment by inducing a similar state in them, cells were injected intravenously following a 2-week period of treatment with 6-MP. Dose levels of 10 mg./kg./day (10 rabbits), 12 mg./kg./day (10 rabbits), and 18 mg./kg./day (14 rabbits) were used. Spleen donor and recipient animals were alternated between Dutch and white New Zealand strains. One week after spleen cell injection the animals were cross-skin-grafted and the survival period noted. A third group of 14 animals was treated with 18 mg./kg. 6-MP, starting on the day of grafting, and spleen cells were injected after 2 weeks of treatment. In no group was a significant prolongation of skin homograft survival noted, nor was the mortality from treatment appreciably affected. TABLE 3 summarizes the observations made in this experiment.

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* From Armour Laboratories, Chicago, Ill.

TABLE 3
SPLEEN CELL INJECTION AND 6-MP TREATMENT

Total no. animals	No. surviving treatment	WBC after treatment	
44	33	Av. (27) = 4600 (1050–8300)	
No. spleen cells injected and percentage viable	No. surviving to grafting	Av. slough time of homografts	
		Treated	Not treated
Av. (31) = 3.07×10^8 80% ($0.01–320 \times 10^8$) (40–95)	29	17.1 (10–21)	15.6 (10–26)

TABLE 4
TREATMENT STARTING 7 DAYS AFTER GRAFTING

	Number	Mean slough time	Range	Per cent mortality
Treated	8	14.1 days (3 animals)	10–21	62.5*
Nontreated	8	13.6 days	10–17	0

* Two animals died at 14 days, 1 animal at 17 days, and 1 animal at 18 days with completely viable grafts, and 1 animal died at 15 days with a sloughing graft.

Effect of starting treatment after grafting. This experiment was designed to evaluate the effect of 6-MP on the survival of skin homografts when treatment was started after the homograft response had been initiated. At 7 days postgrafting the homograft is in good condition, but the data of Converse *et al.*[19] indicate that at this time a state of immunity exists in the rabbit, which is demonstrated by accelerated rejection of second-set homografts.

The rabbits in this experiment were treated with a dose of 10 mg./kg. 6-MP, starting 7 days after grafting, and the treatment was continued until slough occurred. As can be seen from TABLE 4, the mortality due to treatment was again excessive. Although 3 animals sloughed their grafts in the same time period as controls, 4 animals had completely viable grafts at the time of death. These animals were also ill with diarrhea and exhibited marked dehydration and weight loss 2 or 3 days prior to death. If the animals had survived their treatment, the homograft survival period might have been prolonged over that of controls. Although there was no marked effect of 6-MP on homograft survival, the possibility of some prolongation is not completely eliminated. Experiments are in progress to establish this point definitely and will be reported.

Effect of 6-MP on survival of second-set homografts. Fourteen rabbits (7 pairs) received second-set homografts 2 weeks after slough of the primary homograft. Eight animals were treated with 18 mg./kg./day of 6-MP, beginning on the day of second-set homografting and continuing until slough. Homograft survival in the treated animals averaged 9.25 days (8 to 12), contrasted with 10.2 days (9 to 12) in the untreated animals. Thus, at the dose level used, no effect on second-set homograft survival was noted.

Effect of 6-MP on Skin Homograft Survival in Mice

Parallel studies on the effect of 6-MP on skin homograft survival were conducted, using inbred mice. Inbred female mice of strains B_1, C, and BALB/c from a colony maintained by J. B. Aust of the University of Minnesota Medical School were used in all studies to be reported. Preliminary studies revealed that the mean slough time of skin homografts exchanged between B_1 and C strains as determined by daily gross observation was approximately 7 days. Toxicity studies revealed that 6-MP doses as high as 150 mg./kg./day intraperitoneally were tolerated with a low mortality (30 per cent or less) for a period of 10 days. The following treatment groups were set up:

Group I consisted of 14 B_1 and 14 C mice treated with a dose of 72 mg./kg./day I.P. Treatment was started on the day reciprocal full-thickness skin grafts were exchanged between strains. Skin grafts were also reciprocally exchanged between a group of 20 B_1 and 20 C untreated mice that served as controls.

Group II consisted of 20 B_1 and 20 C mice treated with a dose of 100 mg./kg./day 6-MP, starting on the day of grafting. Skin homografts were exchanged between a control group of 20 untreated B_1 mice and 20 untreated C mice.

Group III consisted of 10 B_1 and 10 C mice treated with a dose of 150 mg./kg./day, beginning on the day of reciprocal cross-grafting. A similar control group of 10 untreated B_1 and 10 untreated C strain mice were reciprocally cross-grafted. TABLE 5 summarizes the results of these experiments, which failed to provide evidence that 6-MP treatment prolongs skin homograft survival in mice.

Other experiments have been conducted to evaluate the effect of treatment prior to grafting with single doses of 600 mg./kg./day 6-MP on the survival of skin homografts grafted from BALB/C mice to B_1 mice. Only 2 survivors were obtained of 20 treated mice, and homografts in these mice sloughed at a time comparable to that of controls. The experiments were repeated, and spleen cells from animals of the skin graft donor strain suspended in buffered Hanks' balanced salt solution were administered intravenously in an effort to protect the treated mice from lethal effects of 6-MP that might result from marrow suppression. The equivalent of 1 whole donor spleen from an animal of the prospective donor strain was injected intravenously in a group of 10 animals the day after a single 600-mg./kg. dose of 6-MP was given and daily for 4 days following the same dose of 6-MP in another group of 10 animals. The mortality was identical (18 deaths of 20 animals) as that observed in the group of mice not receiving spleen cells. No prolongation of graft survival was noted in the 2 survivors. Lower doses of 6-MP (150 to 400 mg./kg.) in single or multiple daily doses up to 10 days, combined with single and multiple daily doses of spleen cell suspension, while providing an increased number of survivors, have failed to alter the survival time of skin homografts. Spleen cell injection alone in a dose of the suspended cells from 1 spleen per injection given as a series of daily injections as long as 10 days was well tolerated (2 deaths in 10 animals). Homografts in this group showed a slight prolongation, but with a mean slough time of 22.5 days (17 to 27) in treated animals and a mean slough time of 17.6 days (15 to 23) in controls. These results were not significantly different.

TABLE 5
EFFECT OF 6-MP TREATMENT STARTING WITH GRAFTING ON MOUSE SKIN HOMOGRAFT SURVIVAL

Group	Strain combination	Dose	Mean slough time (days)	Per cent mortality
I Treated	B_1 to C	72 mg./kg./day	12	0
	C to B_1		12	
Control	B_1 to C		8	0
	C to B_1		7	
II Treated	B_1 to C	100 mg./kg./day	9	0
	C to B_1		12	
Control	B_1 to C		7	0
	C to B_1		11	
III Treated	B_1 to C	150 mg./kg./day	7	15
	C to B_1		8	
Control	B_1 to C		7	0
	C to B_1		7	

Experience with Other Antimetabolites

Because of success with 6-MP in achieving prolongation of skin homograft survival other purine analogues and agents known to interfere with nucleoprotein metabolism were studied to ascertain whether they might have similar effects on the homograft reaction. Two agents in particular were chosen because of earlier reports dealing with their ability to suppress the immune response. One of them, 8-azaguanine, has been shown to interfere with antibody synthesis both *in vivo*[20] and *in vitro*.[21] Uphoff[22] found that a significant number of mice that would normally succumb from a homograft reaction following lethal total body X irradiation and homologous marrow transfusion could be spared if treated with A-Methopterin. Other agents tested included 6-methylmercaptopurine, 5-fluorouracil, and 6-aminonucleoside of Puromycin.* In no case have any of these agents produced prolongation of skin homograft survival with the treatment schedules used. Our experience with these drugs is summarized in TABLE 6.

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* Trade-mark of 6-dimethylamino-9-(3′-deoxy-3′-p-methoxy-L-phenylanylamino)-Bd-ribofuranosyl)purine.

TABLE 6

EFFECT OF OTHER ANTIMETABOLITES ON SKIN HOMOGRAFT SURVIVAL

Antimetabolite	Treatment schedule	Animal	Mortality	Bone marrow suppression	Effect on homograft survival
6-Methyl mercaptopurine	12 mg./kg./day I.V. 25 mg./kg./day I.V. Starting with grafting to slough	Rabbit	Low Low	Slight Slight	None
8-Azaguanine	10 mg./kg./day I.V. Starting with grafting to slough	Rabbit	High	—	None
Aminonucleoside	1.5 mg./100 gm. body wt. × 6 days S.C. 0.5 mg./100 gm. body wt. × 6 days S.C. Prior to grafting	Rat	Low Low	—	None
A-Methopterin	3 mg./kg./day I.V. 6 mg./kg./day I.V. Starting with grafting to slough	Rabbit	Low Moderate	Slight Slight	None
5-Fluorouracil	5 mg./kg./day I.V. 12 mg./kg./day I.V. Starting with grafting to slough	Rabbit Rat	High Low	— —	None None

Discussion

The data presented establish that 6-MP given in doses of 6 mg./kg./day and higher produce prolongation of skin homograft survival in rabbits. The data on mouse skin homograft survival provide evidence that the effect obtained may be limited to certain species. Because of the toxicity associated with the high doses used, the potential maximal prolongation of skin homograft survival was not achieved. A number of animals died with viable skin homografts at a time period well beyond the slough time of untreated animals. It also should be pointed out that an individual variation in susceptibility to the effect of 6-MP was noted. In all treated groups there were some animals that sloughed their grafts at a time comparable to that of the controls. This variability of action extended also to the bone marrow-suppressive effect of 6-MP. There appeared, however, to be no positive correlation between the degree of leukopenia achieved and the condition of the skin homografts.

The increased toxicity of 6-MP in ungrafted animals when compared with grafted animals is of some interest. This finding has been noted by other workers in this laboratory studying the effect of 6-MP in other phases of the immune response (Hoyer, Condie, and Good; unpublished observations). That this protective effect may be due to a stimulation of the adrenal cortex by anesthesia and surgery has been considered, but it seems unlikely in view of the high mortality obtained in experiments in which steroids were given together with 6-MP (Meeker, unpublished observations). A nonspecific stimulation of the reticuloendothelial, lymphoid, and hematopoietic systems by the homograft seems likely as the best explanation for the differences in mortality. We (Good and Stetson, unpublished observations) and others[23] have observed that injection of endotoxin may exert a protective effect on the lethal action of nitrogen mustard and irradiation, and a similar protective stimulation may be produced by the skin homograft in animals treated with 6-MP.

The time at which treatment is instituted appears to be an important factor in evaluating the effect of 6-MP and related drugs on the homograft response. This concept is also supported by the data of Schwartz *et al.*,[1-3] who worked with a simple protein antigen. Although the data on treatment beginning after grafting at a time when the homograft response should be well established[19] are preliminary, the data on pretreatment indicate that 6-MP has no effect on the homograft response subsequently elicited. It appears that treatment must be started at the time of grafting and also must be continuous in order for the protective effect of 6-MP to be demonstrable.

The species of animal used appears to be an important determinant of the prolongation of skin homograft survival. Studies in mice failed to reveal any evidence for prolongation of skin homograft survival in a large number of experiments using a wide range of dosages. The reason for this failure is not apparent at this time. Prolongation of homograft survival also appears to be a property possessed only by 6-MP among the group of compounds studied. Several of the other compounds have been reported to suppress the formation of classic antibody, but none was noted to have any effect on the homograft reaction. All compounds tested were thought to be potent inhibitors of nucleoprotein synthesis.

A final point to be considered is the mechanism of action by which 6-MP prolongs the survival of skin homografts. In general, one may postulate 2 mechanisms: (1) a direct metabolic interference with the homograft rejection mechanism, or (2) an indirect effect operating through the cytotoxic action of this drug. We feel that the evidence from these and other experiments favors the first mechanism.

In experiments with nitrogen mustard, utilizing doses sufficient to produce profound bone marrow suppression, lymphoid atrophy, and cachexia, we were unable to note any effect on homograft survival.[24] Although starvation has been reported to depress the immune response,[25] this was accomplished only after a prolonged period. Neither do we consider that vitamin deficiency[26] represents a probable cause for the prolongation of skin homograft survival, because the time periods involved were generally too short for development of a deficiency state. Additional evidence that the toxic effects were not essential for prolonged survival was provided by the experiments in which 6-MP was administered for 2 weeks after grafting and then discontinued. Little evidence of toxicity was demonstrated, but a prolongation of skin homograft survival still occurred that was of the same order of magnitude as that obtained in groups where treatment produced cachexia.

The exact biochemical mechanism by which 6-MP acts on the homograft reaction is unclear. In general, 6-MP appears to act as an antagonist to nucleic acid metabolism. Numerous studies[27-29] have revealed several metabolic pathways by which it may inhibit nucleic acid synthesis. Interference with enzymes of carbohydrate metabolism also has been demonstrated.[30,31] Definitive studies are necessary to determine whether any of the known metabolic effects of 6-MP are important factors in the suppression of the homograft response. A possible approach is to attempt a reversal of the 6-MP effect on the immune response by simultaneously administering the naturally occurring purines with which 6-MP is known to compete in biological systems.

Summary

Experiments have been outlined that demonstrate that 6-MP exerts a suppressive effect on the homograft rejection mechanism. This effect is dependent upon the dose employed and the time at which treatment is given. A species specificity has also been demonstrated. This homograft-suppressive property has not been demonstrated for other closely related drugs. It is of interest that alkylating agents, which produce profound leukopenia, have not been demonstrated by us to prolong skin homograft survival in the same system used for the evaluations presented in this study. Hence bone marrow suppression by 6-MP does not appear to be an important factor in the homograft prolongation noted; neither does the general toxicity of the drug appear to be important in skin homograft prolongation, as the other drugs tested were equally toxic. It is believed that prolongation of skin homograft survival produced by 6-MP occurs through an inhibition of metabolic processes vital to the host's immune response to foreign tissues.

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References

1. SCHWARTZ, R., J. STACK & W. DAMESHEK. 1958. Effect of 6-mercaptopurine on antibody production. Proc. Soc. Exptl. Biol. Med. **99:** 164.
2. SCHWARTZ, R., J. EISNER & W. DAMESHEK. 1959. The effect of 6-mercaptopurine on immune responses. Clin. Research. **7:** 39.
3. SCHWARTZ, R. 1959. The effect of 6-mercaptopurine on primary and secondary immune responses. J. Clin. Invest. **6:** 1041.
4. GORER, P. A. 1958. Some reactions of H-2 antibodies *in vitro* and *in vivo*. Ann. N. Y. Acad. Sci. **73**(3): 707.
5. GOOD, R. A., R. L. VARCO, J. B. AUST & S. ZAK. 1957. Transplantation studies in agammaglobulinemia. Ann. N. Y. Acad. Sci. **64**(5): 882.

6. Stetson, C. A., Jr. & R. Demopoulos. 1958. Reactions of skin homografts with specific immune sera. Ann. N. Y. Acad. Sci. **73**(3): 687.
7. Goodman, L. S. & A. Gilman. 1958. The pharmacological basis of therapeutics. 2nd. ed. : 1432. Macmillan. New York, N. Y.
8. Algire, G. H., J. M. Weaver & R. T. Prehn. 1957. Studies on tissue homotransplantation in mice, using diffusion chamber methods. Ann. N. Y. Acad. Sci. **64**(5): 1009.
9. Mitchison, N. A. 1954. Passive transfer of transplantation immunity. Proc. Roy. Soc. London. **B142:** 72.
10. Billingham, R. E., L. Brent & P. B. Medawar. 1954. Quantitative studies on tissue transplantation immunity. II. The origin, strength and duration of actively and adoptively acquired immunity. Proc. Roy. Soc. London. **B143:** 58.
11. Brent, L. 1957. Transplantation immunity and hypersensitivity in mechanisms of hypersensitivity. Henry Ford Hospital International Symposium. : 555. Little, Brown. Boston, Mass.
12. Meeker, W., R. Condie, D. Weiner, R. L. Varco & R. A. Good. 1959. Prolongation of skin homograft survival in rabbits by 6-mercaptopurine. Proc. Soc. Exptl. Biol. Med. **102:** 459.
13. Egdahl, R. H., R. A. Good & R. L. Varco. 1957. Studies in homograft and heterograft survival. Surgery. **42:** 228.
14. McQuarrie, D. G., J. H. Kim & R. L. Varco. 1959. Long term survival of intrastrain homografts in commercially bred rats. Transplantation Bull. **6:** 97.
15. Taylor, C. A. & J. W. Lehrfeld. 1955. Definition of survival time of skin homografts. Ann. N. Y. Acad. Sci. **59**(3): 351.
16. Clarke, D. A., F. S. Philips, S. S. Sternberg, C. C. Stock, G. B. Elion & G. H. Hitchings. 1953. 6-mercaptopurine: effects in mouse sarcoma 180 and in normal animals. Cancer Research. **13:** 593.
17. Trentin, J. J. 1957. The immunological basis for induced tolerance to skin homografts in irradiated mice receiving bone marrow transfusions. Transplantation Bull. **4:** 74.
18. Wilson, R. E., J. B. Dealy, N. T. Sadowsky, J. M. Corson & J. E. Murray. 1959. Transplantation of homologous bone marrow and skin from common multiple donors following total body irradiation. Surgery. **46:** 261.
19. Converse, J. M., D. L. Ballantyne & J. Woisky. 1958. The vascularization of skin homografts and transplantation immunity. Ann. N. Y. Acad. Sci. **73**(3): 693.
20. Malmgren, R. A., B. E. Bennison & T. W. McKinley. 1952. The effect of guanazolo on antibody formation. J. Natl. Cancer Inst. **12:** 807.
21. Dutton, R. W., A. H. Dutton & M. George. 1958. Effect of 8-azaguanine on antibody synthesis *in vitro*. Nature. **182:** 1377.
22. Uphoff, D. E. 1959. Alteration of homograft reaction by a-methopterin in lethally irradiated mice treated with homologous marrow. Proc. Soc. Exptl. Biol. Med. **99:** 651.
23. Smith, W. W., I. M. Alderman & R. E. Gillespie. 1958. Hematopoietic recovery induced by bacterial endotoxin in irradiated mice. Am. J. Physiol. **192:** 549.
24. McQuarrie, D. G., R. M. Condie, W. R. Meeker, F. R. Roller & R. L. Varco. 1960. Effect of methyl bis(2-chlorethyl) amine upon survival of skin homografts in rats and rabbits. Proc. Soc. Exptl. Biol. Med. In press.
25. Cannon, P. R., W. E. Chase & R. W. Wissler. 1943. The relationship of the protein-reserves to antibody-production I. The effects of a low-protein diet and of plasmapheresis upon the formation of agglutinins. J. Immunol. **47:** 133.
26. Fisher, B., A. E. Axelrod, E. R. Fisher, S. H. Lee & N. Calvanese. 1958. The favorable effect of pyridoxine deficiency on skin homograft survival. Surgery. **44:** 149.
27. Davidson, J. D. & B. B. Freeman. 1955. The effects of antitumor drugs upon P_{32} incorporation in nucleic acids of mouse tumors. Cancer Research. **15:** 31.
28. Skipper, H. E. 1955. On the mechanism of action of 6-mercaptopurine. Ann. N. Y. Acad. Sci. **60**(2): 315.
29. Elion, G. B. & G. H. Hitchings. 1953. The purine metabolism of a 6-mercaptopurine-resistant *Lactobacillus casei*. J. Biol. Chem. **204:** 35.
30. Hochstein, P. 1957. Synergistic effects of 6-mercaptopurine and methotrexate on anaerobic glycolysis by subcellular fractions of S91 mouse melanoma. Proc. Am. Assoc. Cancer Research. **2:** 214.
31. Burk, D., P. Hochstein, J. Hunter, B. Landau, S. Lazlo, K. Wight & M. Woods. 1958. A rationale of cancer chemotherapy based on primary inhibition of glucose phosphorylation (hexokinase reaction) at substrate, coenzyme, and enzyme levels. Acta Unio Intern. contra Cancrum. **14:** 66.

THE INFLUENCE OF EXPERIMENTALLY INDUCED CHANGES IN THE LOCAL ENVIRONMENT OF GRAFTED CELLS UPON THE ESTABLISHMENT AND GROWTH OF HOMOLOGOUS AND HETEROLOGOUS TUMOR TRANSPLANTS

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The absence or inhibition of a host's immunity reactions is a necessary but not sufficient condition for the successful establishment of normal and neoplastic grafts. Other factors such as adequate local environment and the development of stromal reactions obviously are necessary for this process. Various factors influencing immunity to transplanted tumors have been studied and discussed by many authors. However, there have been only a few investigations dealing with the effect of changes in local environment upon the fate of grafts. The following discussion of some of the aspects of this problem is based on experimental data of my own.

Numerous facts indicate that aseptic inflammation and connective tissue proliferation favor the establishment and growth of tumors (Vasiliev, 1958). Transplantation into the "air pouch" (method of Selye and Horava) is a method that may be used for the stimulation of such local reactions around grafted cells. Our experiments with 4 different strains of transplantable rat and mouse neoplasm have shown that the rate of growth of isologous and homologous tumors grafted in the air pouch is three or four times that of the same tumors grafted subcutaneously. A similar stimulation of tumor growth in air pouches was observed in experiments with human and mouse neoplasms heterologously grafted to untreated or cortisone-treated rats.

It may be inferred that conditions existing in the inflamed area favor the growth not only of homologous but also of heterologous tumor cells.

The influence of the local environment of tumor cells has been studied also in experiments with grafts consisting of neoplastic and normal cells combined. Such grafts are similar in many respects to combined explants studied by Leighton (1957) and other authors. The first experiments indicating that embryonic tissue graft may stimulate the growth of simultaneously transplanted tumor were described by Greene (1957) and by Schneyer (1955).

My associates and I have studied the effect of embryonic tissue suspensions on the growth of different isologous, homologous, and heterologous tumors: Ehrlich ascites tumor, Crocker sarcoma 180, mammary carcinoma of C3HA mice (RSM tumor), mammary carcinoma of A mice (MAP tumor), sarcoma 298 of C57BL mice, and rat sarcoma 45.* It was shown that embryonic suspensions cause a significant stimulation of growth of all these neoplasms. The stimulating effect was observed only in experiments in which the quantity of embryonic tissue injected into each animal was many times that of the tumor tissue.

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* Suspensions were prepared by mincing the whole body of an 18- to 20-day embryo. All the conclusions mentioned in this paper are based on statistically significant changes over those of controls in the mean weight of tumor or in the mean longevity of animals after tumor implantation.

Embryonic tissue acts locally: it has no stimulating effect when injected at a site other than the site of tumor implantation.

Embryonic tissue suspensions increase the rate of tumor growth only when injected simultaneously with neoplastic tissue: isologous embryonic tissue grafted intraperitoneally 12 days before or 15 days after the transplantation of Ehrlich tumor cells had no effect upon the growth rate of these cells.

Embryonic tissue killed by heating up to 60° C. or by repeated freezing to −50° C. lost its activity. This growth-stimulating activity remained unchanged, however, when embryonic tissue was kept 10 to 20 days at +4° C.

Growth-stimulating activity depends on the species specificity of the embryonic tissue used: in all experiments, regardless of the species specificity of the grafted tumor cells, suspensions of the embryos homologous to the host were the most active. For instance, in experiments with homologously grafted mouse sarcoma 180, suspensions of mouse embryos proved to be most active and in experiments with the same tumor heterologously grafted to rat tissue, the embryos had the greatest activity. In experiments with isologously grafted sarcoma 298 of C57BL mice, isologous tissue of the embryos of the same mouse strain was more active than the tissue of embryos of other mouse strains. Such strain specificity of the growth-stimulating effect of embryonic tissue was not observed, however, in experiments with 2 other mouse tumors (mammary carcinomas MAP and RSM).

Suspensions of various normal tissues of adult homologous and heterologous animals have a very weak stimulating ability or none at all.

An analysis of the facts revealed in the experiments with embryonic tissue leads to the conclusion that the effect of these tissues on the growth of transplanted tumors is different in its characteristics from the well-known stimulating effect of embryo extracts in tissue cultures. The effect of embryo suspension has nothing in common with the "conditioning effect" of lyophilized tissues (the so-called Kaliss effect, XYZ effect, and related phenomena). It seems probable that growth stimulation is associated with the presence of living embryonic cells at the site of tumor implantation. The growth-stimulating activity of embryonic cells is similar in its characteristics to that of tumor cells destroyed by X rays (Révész, 1958). However, the nature of the growth-stimulating substances produced by embryonic and tumor cells remains unknown.

The effect of embryonic tissues upon the growth of tumor cells in peritoneal fluid is interesting as regards the question of the nature of cell changes during the conversion of neoplasm into an ascitic form.

In experiments with Puyman's strain of lymphatic leukemia of C57 mice (La strain) an accumulation of ascitic fluid after intraperitoneal grafting of this neoplasm was observed, but the neoplastic cells did not multiply actively in this fluid. Numerous efforts at establishing the neoplasm in the ascitic form by intraperitoneal passages of ascitic fluid gave negative results. Thus, after Klein's classification (G. Klein, 1956), the La strain may be regarded as a neoplasm inconvertible into the ascitic form.

When suspensions of isologous embryonic tissue were added to the intraperitoneally transplanted material, the La cells in the ascitic fluid multiplied faster: the concentration of neoplastic cells in the ascitic fluid was several times as great as in the controls, into which embryonic tissue had not been transplanted. The use of embryonic tissue suspensions made possible the serial passages of La strain by ascitic fluid. Thirty-five serial passages were made; embryonic tissue was added to the transplanted ascitic fluid in each passage.

Even after such a series of transplantations the La cells did not acquire an ability to grow without stimulation in ascitic fluid: each time an effort was made to maintain the neoplasm by transfers of the ascitic fluid alone, the multiplication of the neoplastic cells slowed down and, at length, after one or two such transfers, the results of grafting became invariably negative.

It may be concluded that even after numerous intraperitoneal passages the growth of La cells in the ascitic fluid remained dependent on stimulating factors supplied by the embryonic tissue. The situation may be compared with that observed in experiments with "hormone-dependent" neoplasms, which grow only in the presence of excessive quantities of certain hormones.

Several suggestions concerning changes in cell physiology associated with conversion into ascitic form may be made on the basis of the experiments with the La strain. It seems possible that cells of ascitic tumors are so able to change their environment, that of peritoneal fluid, as to cause certain substances that stimulate the growth of these cells to accumulate in such fluid. This suggestion agrees well with the results of our earlier published experiments (Vasiliev, 1951), which showed that cell-free filtrate of the ascitic fluid accumulated after the implantation of Ehrlich carcinoma favors the establishment and growth of cells of the same neoplasm.

Cells of "solid" tumors probably are not able to induce an accumulation of growth-stimulating substances in peritoneal fluid; these cells are not able to multiply actively in such fluid unless stimulating substances are supplied from an external source.

Ascitic tumors may be regarded as tumors that become independent of a supply of growth-stimulating factors, just as hormone-dependent tumors may become hormone-independent in the course of their growth. New experiments obviously are necessary to test the validity of this hypothesis.

References

1. Greene, H. S. N. 1957. Heterotransplantation of tumors. Ann. N. Y. Acad. Sci. **69**(4): 818–829.
2. Leighton, J. 1957. Contributions of tissue culture studies to an understanding of the biology of cancer: a review. Cancer Research. **17**: 929–941.
3. Révész, L. 1958. Effect of lethally damaged tumor cells upon the development of admixed viable cells. J. Natl. Cancer Inst. **20**: 1157–1186.
4. Schneyer, C. A. 1955. Effect of normal tissue inocula on homologous tumor transplants. Cancer Research. **15**: 268–270.
5. Vasiliev, J. M. 1958. The role of connective tissue proliferation in invasive growth of normal and malignant tissues: a review. Brit. J. Cancer. **12**: 524–536.

THE SPECIFICITY OF SKIN HOMOGRAFT REJECTION IN MAN*

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In the course of a study of the transfer of delayed hypersensitivity to skin homografts in man with leukocyte extracts,[1] it was noted that the specificity of homograft responses had not been evaluated as extensively in man[2-5] as in animal species.[6-10] This report describes an attempt to demonstrate the individual-specific character of skin homograft rejection in man on the basis of the experimental end point provided by the "white-graft" reaction.

The application of a skin homograft to a human recipient results in its initial vascularization and proliferation, followed within 8 to 12 days by graft vessel thrombosis, hemorrhage, and escharification.[11] These changes are associated with development in the host of generalized altered reactivity to subsequent skin homografts from the same donor source. If such a second-set homograft is applied during the first 7 days following first-set rejection, the white-graft reaction is elicited.[12] If the latent period after first-set rejection is lengthened to 10 to 15 days, the second-set graft exhibits the accelerated-rejection reaction.[13] Accelerated rejection is associated with initial graft vascularization, followed by rapid vascular thrombosis and breakdown, with gross rejection on postoperative days 4 or 5. In contrast, the white-graft reaction is characterized by a complete absence of vascularization on stereomicroscopic observation; the graft remains parchment white and opaque. This sharp end point, which denotes a prior specific immunological experience when contrasted with the behavior of control homografts, has been selected for the present study of homograft specificity in man.

Materials and Methods

Selection of donors and recipients. Skin homograft donors and recipients were volunteer members of the House Staff of the Third (NYU) Medical Division of Bellevue Hospital and medical students at New York University College of Medicine. Nine recipients were selected. The skin homograft donors were all pedigreed blood donors known not to transmit serum hepatitis.

Experimental design. Each recipient was sensitized with a skin homograft from one donor (A). The behavior of this graft was followed from initial vascularization to rejection time. Within 1 to 5 days after rejection each recipient was rechallenged with a graft from the same donor A (test graft) and with a graft from another donor, B (control graft). The behavior of all grafts was followed during the ensuing 2 weeks.

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Surgical technique and method of observation. The method of application of experimental skin homografts in man, as well as the methods of examination and criteria for homograft rejection, have been described in detail in a previous report.[11] Briefly, full-thickness skin grafts 11 mm. in diameter were excised from the donors and applied to the volar surface of the forearms of the recipients. The recipients received the first graft on one arm and were then challenged with test and control grafts applied to the other arm. All grafts were followed daily with gross and stereomicroscopic observations.

Criteria of homograft rejection. The criteria for graft rejection included the development of erythema and edema in the area surrounding the graft, cyanosis and edema of the graft, cessation of blood flow, and thrombosis and hemorrhage in the graft vessels. In each case these events were followed by change of the graft into a black eschar that was subsequently sloughed.

TABLE 1

THE SPECIFICITY OF SKIN HOMOGRAFT REJECTION IN MAN

Recipient	Survival time of sensitizing first-set graft from *A* (days)	Time of subsequent challenge after rejection of graft from *A* (days)	Results of second challenge	
			Behavior of second-set graft from *A*	Survival time of control graft from *B* (days)
Smi.	8	5	White graft	5
Ros.	12	1	White graft	6
Pal.	10	3	White graft	9
Dou.	12	2	White graft	—*
Bur.	10	1	White graft	8
Fle.	11	1	White graft	9
Bas.	10	3	White graft	6
Gan.	10	3	White graft	5
Bra.	11	2	White graft	6

* Technically unsatisfactory.

Grafts undergoing the white-graft reaction were characterized by (1) immediate and marked surface pallor, which persisted throughout the course of the reaction, (2) surrounding erythema and edema of host tissues, (3) complete and permanent absence of stereomicroscopic evidences of vascularization, and (4) subsequent graft opacification, with eventual change of the graft into a tan eschar (post-operative day 6).

Observations

The results of this study are summarized in TABLE 1. The first-set skin homografts (sensitizing grafts) in every recipient were rejected during postoperative days 8 to 12. Each graft was fully vascularized prior to rejection.

Upon subsequent challenge of the recipients with a graft from the sensitizing donor (test graft) and with a graft from a new donor (control graft) within 5 days after rejection of the first-set graft, it was noted that the test grafts exhibited the white-graft reaction in each of 9 consecutive instances. By contrast, the control grafts became fully vascularized and went on to exhibit the classic manifestations of first-set homograft rejection. It is of interest, however, that 5 of the 8 control grafts exhibited a decreased survival period (5 or 6 days). The remaining 3 control grafts were rejected at a time consistent with the usual behavior of first-set grafts in man (8 or 9 days).

Discussion

The prompt and unequivocal end point of specificity selected for this study, namely the white-graft reaction, has been elicited in 9 consecutive instances. However, while all control homografts were vascularized and underwent the usual rejection changes, 5 of the 8 grafts exhibited a significant decrease in survival time (5 or 6 days). This decrease may have been due to a sharing of some tissue transplantation antigens by the donor of the sensitizing graft and the donor of the subsequent control graft. While such a sharing of antigenic determinants was insufficient to cause the control grafts to behave like white grafts, it might have contributed to the earlier rejection of some of the controls.

This study suggests that in man there is a high degree of individual-specific reactivity to skin homografts, when this reactivity is measured as an all-or-none phenomenon such as the experimental model of the white-graft reaction. However, these results do not exclude the possibility that, when homograft reactivity is measured in a different parameter (accelerated rejection, for example), this individual specificity may become obscured by unpredictable chance donor combinations that provide an occasional instance of antigenic overlap.

The advantage of using the white-graft reaction in specificity studies lies in the fact that it is dependent on absolute criteria rather than on numerical comparisons of survival times. For this reason it would appear that the white-graft reaction affords distinct advantages for further studies of altered host reactivity to tissue transplants.

Summary

A high degree of individual-specific reactivity to skin homografts is present in man when this reactivity is evaluated by the experimental model of the white-graft reaction.

References

1. Lawrence, H. S., F. T., Rapaport, J. M. Converse & W. S. Tillett. 1960. Transfer of delayed hypersensitivity to skin homografts with leucocyte extracts in man. J. Clin. Invest. **39:** 185.
2. Baxter, H. & M. A. Entin. 1951. Clinical study of the fate of homografts in man. Effect of repeated dosage from the same donor and of refrigeration on these grafts. Am. J. Surg. **81:** 285.
3. Davis, J. S. 1910. Skin transplantation with a review of 550 cases at the Johns Hopkins Hospital. Johns Hopkins Hosp. Bull. **15:** 307.
4. Gibson, T. & P. B. Medawar. 1942. The fate of skin homografts in man. J. Anat. **77:** 299.
5. Longmire, W. P., H. B. Stone, A. S. Daniel & C. D. Goon. 1947. Report of clinical experiences with homografts. Plastic and Reconstructive Surg. **2:** 419.
6. Gorer, P. A. 1959. Some recent work on tumor immunity. Advances in Cancer Research. **4:** 149.

7. Medawar, P. B. 1954. General problems of immunity. *In* Preservation and Transplantation of Normal Tissues. Ciba Foundation Symposium. Little, Brown. Boston, Mass.
8. Medawar, P. B. 1959. Reactions to homologous tissue antigens in relation to hypersensitivity. *In* Cellular and Humoral Aspects of the Hypersensitive States. : 504. H. S. Lawrence, Ed. Hoeber. New York, N. Y.
9. Billingham, R. E., L. Brent & P. B. Medawar. 1954. Quantitative studies on tissue transplantation immunity. II. The origin, strength and duration of actively and adoptively acquired immunity. Proc. Roy. Soc. London. **B143:** 58.
10. Billingham, R. E., L. Brent & P. B. Medawar. 1956. Quantitative studies on tissue transplantation immunity. III. Actively acquired tolerance. Phil. Trans. London. **B239:** 357.
11. Converse, J. M. & F. T. Rapaport. 1956. The vascularization of skin autografts and homografts. Ann. Surg. **143:** 306.
12. Rapaport, F. T. & J. M. Converse. 1958. The immune response to multiple-set skin homografts. Ann. Surg. **147:** 273.
13. Rapaport, F. T. & J. M. Converse. 1957. Observations on immunological manifestations of the homograft rejection phenomenon in man: the recall flare. Ann. N. Y. Acad. Sci. **64**(5): 836.

Discussion of the Paper

Chandler A. Stetson: Lawrence has done a very good job of setting this particular type of homograft reaction into proper perspective. The "white graft" reaction is one of the least known manifestations of transplantation immunity, and it has been studied less than either the first-set or the typical second-set accelerated rejection.

We have been working with this sort of reaction in experimental animals for some time and have come to very much the same conclusions: namely, that a specifically immunized rabbit will exhibit white-graft reactivity when tested with a skin graft from the donor and frequently will also show accelerated or second-set type of reactivity to skin grafts from apparently unrelated rabbits who nevertheless must share transplantation antigens with the donor.

There are two points that I must raise. What are Lawrence's views as to the place of the white-graft reaction in the general realm of transplantation immunity? There are some reasons for thinking that this is the most highly developed or vigorously expressed manifestation of transplantation immunity.

The reaction is seen pre-eminently when the second graft is applied at what we know is the height of the rejection reaction to the first graft. This is the very time at which one might look for the highest expression of transplantation immunity.

The other point that I raise is the fact that, as compared to first-set and second-set reactions, we know very little about the mediation of the white-graft response. Unlike second-set reactions or accelerated reactivity, it has not been reported in the literature, as far as I am aware, that this kind of reactivity can be transferred adoptively or passively except with hyperimmune serum. Have the authors done any experiments along these lines?

Milton D. Edgerton (*Dept. of Plastic Surgery, Johns Hopkins Hospital, Baltimore, Md.*): It has been interesting to me that in the monographs published in the last four to six years we have continued to return to a very fundamental issue concerning these skin homografts: have these grafts truly persisted? Also, how do we know it in an accurate way?

I think the remarks of Kelly are very interesting to us and must still cause us some concern, because as yet we do not know the exact details of this phenomenon of creeping substitution. We have seen it repeatedly not only in human grafting but in animal grafting. We have learned within the last year in our laboratory that dermal grafts clearly survive as homografts if they are protected from drying by any simple maneuver.

We also know that tattoo pigment in these grafts will not suffice as a test for viability. The pigment pattern can be picked up clearly and permanently by the replacement or replacing tissue cells. This is also true of pigment naturally occurring in the grafts themselves. A black graft placed on a white animal will frequently allow its pigment to be picked up by white host cells even with complete rejection.

Peacock showed us a very fine study on cartilage and raised again the question of the survival of the noncellular stromal elements in cartilage. He pointed out, very accurately, I think, that dead cartilage will also pick up radioactive sulfur in lesser amounts than viable cartilage.

LAWRENCE: The sequence of the white graft on second-, third-, and fourth-set homografts is, I think, identical. Actually, we became aware of the fact that a white-graft reaction existed during our attempts to transfer homograft sensitivity to man. Ordinarily, three weeks elapsed between each first-, second-, third-, and fourth-set homograft application used to sensitize leukocyte donors actively.

In trying to accelerate this schedule, we shortened the interval between grafting to seven days and then found each graft after the initial one was rejected as a white graft. This phenomenon and its possible significance have been described by Rapaport and Converse.

I think the biological and immunological meaning of the white-graft reaction is open to a great deal of interpretation, and is just beginning to be explored.

May I take a minute to discuss the background of the thinking about white grafts? The white graft was originally observed in *hetero*grafted animals, that is, transplantation of skin from a guinea pig to a rabbit resulted in a white graft. Therefore, when the white graft began to be seen following exchange of skin between individuals of the same species, the stage was set for the concept that the white graft represents a more intense manifestation of homograft hypersensitivity than accelerated rejection.

Since the intensity and tempo of any homograft reaction is conditioned by the genetic disparity between the donor and recipient in terms of how many antigens are shared, the concept evolved that the white graft occurring in a homografted individual represented a more intense form of immune response.

I do not know whether it does. I think, tentatively, from the incomplete information available that the white graft may represent another type of immune response in homografts conditioned by the temporal application of the test material. Philip Gell has suggested that it may result from the operation of serum antibody acting on the capillary buds of the ingrowing vessels.

The facts at hand suggest that leukocyte extracts obtained from individuals sensitized by repeated white grafts are not able to transfer accelerated homograft rejection to another recipient, nor will the white-graft reaction be transferred.

We think this arises because the white graft, as it is never vascularized, does not function as an adequate antigenic stimulus for cellular transfer. We know that at least four grafts are needed to sensitize donor leukocytes for the transfer of systemic sensitivity to the skin graft, and that the second, third, and fourth grafts must undergo accelerated rejection.

In conclusion, I think perhaps the white graft may prove to be either a qualitatively different or a more intense form of immune response against foreign tissue perhaps mediated by serum antibody. Evidence accumulated to date indicates that it does not function as an adequate antigenic stimulus for active sensitization of leukocyte donors to the degree of sensitivity requisite for successful cellular transfer of accelerated homograft rejection.

Part III. Antigen-Antibody Manifestations in Tissue Transplantation

THE TRANSFER OF HOMOGRAFT SENSITIVITY (ACCELERATED REJECTION) WITH DNASE-TREATED LEUKOCYTE EXTRACTS IN MAN*

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Since homograft rejection is an immunological response possessed of an exquisite degree of specificity,[1-6] efforts designed to ameliorate or abolish it are logically dependent upon the precise definition of the antigen(s) that induce homograft sensitivity and the antibody(ies) that mediate it. With such information at hand there are available, or can be fashioned, immunological devices capable of altering favorably the host's acquired antagonism to foreign tissues.

In infection with bacterial cell populations, the relative prominence of serum antibody mechanisms over those of delayed hypersensitivity invoked in the recognition and attempted disposal of foreign cells by the host is conditioned by, and will fluctuate with, the characteristics of the particular bacterium involved; for example, contrast host response to the pneumococcus with that to the tubercle bacillus. There is also evidence to suggest a similar fluctuation of the mechanism instrumental in the rejection of tissue cell populations infecting the host, a fluctuation conditioned in turn by the characteristics of the particular tissues involved; for example contrast the rejection mechanism evoked by dissociated cell populations with that evoked by orthotopic skin homografts.[1,3,4]

With respect to rejection of orthotopic skin homografts there is cogent evidence to implicate cell-bound immune factors as the prime instruments of graft destruction[7-13] and conventional serum antibody as an ancillary agent[4,14-16] or as a parallel, yet perhaps unrelated, immunological event.[1,17,18]

The cellular transfer system affords the nearest semblance to an antibody-like reagent available for the analysis of the delayed type of allergic inflammatory response.[7,27] In animal species, cellular transfer has proved to be a valuable technique in advancing knowledge concerning delayed allergy of bacterial and simple chemical origin.[19,20] Cellular transfer has been recently applied to an analysis of the mechanism of homograft sensitivity in mice[8-10,17,18] and guinea pigs[11] and has yielded significant clues concerning the immunological nature of this event.

In human species two unique aspects of the behavior of the cellular trans-

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fer system of immediate concern are: (1) The demonstrated effectiveness of leukocyte extracts in the transfer of delayed sensitivity,[21-25] a reality that affords an opportunity for identification of the factor or factors (transfer factor) concerned in mediating this effect, and (2) the fact that viable leukocytes[16,26] or leukocyte extracts,[22] when obtained from the *peripheral blood*, have not been found capable of transferring the capacity for serum antibody formation.

It also would appear that the function transferred by leukocyte extracts to human recipients consists of both the effector reagent of delayed allergy (transfer factor) and the machinery necessary for its continued production in the recipient as in the leukocyte donor.[27,28,29]

We have recently reported on the transfer of skin homograft hypersensitivity (accelerated homograft rejection) in man by means of an injection of desoxyribonuclease (DNase)-treated leukocyte extracts obtained from the peripheral blood of specifically sensitized donors.[30] Since this work has been published in detail, only a brief summary of the results is discussed here.

Methods

The experimental plan for these experiments had the following four stages:

(1) Skin grafts from subject *A* were used to sensitize subject *B*.

(2) Anti-*A* leukocytes were obtained from patient *B*, and a DNase-treated extract was prepared and injected into subject *C*.

(3) Sensitivity transferred to subject *C* was measured by the rejection time of a test graft (skin from *A*) as compared to that of a control graft (skin from *D*) from a subject unrelated to the experiment.

(4) A positive result was scored when the test graft was rejected in an accelerated fashion (4 to 6 days), while the control graft was rejected as a first-set graft (10 to 12 days). Full-thickness (11 mm. in diameter) skin grafts were employed, and the time of rejection was determined by stereomicroscopic and gross criteria of skin homograft rejection described in earlier reports.[31] Leukocytes were isolated in the usual fashion, frozen and thawed alternately for 7 to 10 cycles, and treated with crystalline pancreatic DNase before injection into the recipient.[21,33] The subjects who served as volunteers for this study were physicians and medical students, for the most part. The donors of skin or leukocytes were chosen with the knowledge that they had not transmitted homologous serum hepatitis in the recent past.

Results

It was observed that neither 1 skin homograft (a first-set graft) nor 2 homografts (a first-set subsequently followed by a second-set graft from the same individual) were sufficient to sensitize the individual to the extent that his leukocyte extracts were capable of effecting a systemic transfer of sensitivity; that is, leukocyte extracts injected in the shoulder and test and control homografts applied to forearms.

However, if the sensitized leukocyte extract is injected into the skin of the recipient around the graft halo-fashion,[14] then 2 homografts (a first- and subsequent second-set) are sufficient to effect transfer of *local*, but not systemic, sensitivity. This type of result is summarized in TABLE 1.

TABLE 1
EFFECTS OF LOCAL TRANSFER FROM LEUKOCYTE DONOR SENSITIZED WITH 2 GRAFTS*

Leukocyte donor	Sensitization of leukocyte donor	Type of transfer	Time of transfer	Material used for transfer	Dosage injected around each graft site (ml.)	Recipient No.	Day of graft rejection		Interpretation of results
							Test graft	Control graft	
D4	2 Grafts	Local (pooled WBC first- and second-set rejection)	3 Days after grafts applied	Sensitive WBC extract	0.22	R5	4–5	8	Positive
				Sensitive serum	1.0		10	8	
D5	2 Grafts	Local (pooled WBC first- and second-set rejection)	3 Days after grafts applied	Sensitive WBC extract	0.2	R6	4	10	Positive
				Sensitive serum	1.0	R7	10		Control
				Nonsensitive WBC extract	0.2		10		
D6	2 Grafts	Local (pooled WBC first- and second-set rejection)	3 Days after grafts applied	Sensitive WBC extract	0.17	R8	5–6	10	Positive
				Sensitive WBC extract	0.17	R9	5–6	10	Positive
				Sensitive serum	1.0	R10	10		Control
				Nonsensitive WBC extract	0.20		10		

* Reproduced from Lawrence *et al.*[30] with permission from the *Journal of Clinical Investigation.*

It may be seen that serum obtained from the donor of sensitized leukocyte extract had no effect upon the rejection time of the test graft. Nonsensitive leukocyte extracts did not affect the rejection-time of test grafts, nor did sensitive leukocyte extracts affect the rejection time of control grafts.

In order to effect a *systemic* transfer of homograft sensitivity it was necessary to sensitize the leukocyte donor with a series of 4 successive skin homografts (that is, first-set, second-set, third-set, and fourth-set homografts applied at intervals of 3 or 4 weeks between each graft). The results of the systemic type of transfer are summarized in TABLE 2.

It is of interest that if, in the course of sensitization of the leukocyte donor, the interval between application of the first-set graft and each subsequent graft is foreshortened to 1 week, the second-, third-, and fourth-set grafts will undergo the white-graft reaction of rejection instead of accelerated rejection (*see* Rapaport *et al.* elsewhere in this monograph). Leukocyte extracts obtained from an individual sensitized in this fashion did not transfer either the white-graft reaction or accelerated rejection to nonsensitive recipients.

TABLE 2

EFFECTS OF SYSTEMIC TRANSFER FROM LEUKOCYTE DONOR SENSITIZED WITH 4 GRAFTS VIA ACCELERATED REJECTION REACTION*

Leukocyte donor	Type of transfer	Time of transfer	Material used for transfer	Dosage WBC (ml.)	Recipient No.	Day of homograft rejection		Interpretation of result
						Test graft	Control graft	
D9	Systemic	8 Days before grafts applied	WBC extract	0.7	R22	4	11	Positive
				0.7	R23	4	8	Positive
D10	Systemic	8 Days before grafts applied	WBC extract	0.9	R24	4	13	Positive
				0.9	R25	4	7	Positive

* Reproduced from Lawrence *et al.*[30] with permission from the *Journal of Clinical Investigation.*

A summary of the results of the transfer of specific sensitivity to skin homografts is set out in TABLE 3.

Discussion

The results achieved following transfer of homograft sensitivity with leukocyte extracts generally parallel those obtained in man following similar transfer of delayed bacterial (tuberculin, streptococcal proteins, diphtheria toxoid) and fungal (coccidioidin) hypersensitivities. This finding fulfills a significant criterion relating homograft hypersensitivity to delayed hypersensitivity of the tuberculin type.

The prompt onset of accelerated rejection within 24 to 48 hours following transfer to recipients bearing fully vascularized homografts may be taken as good evidence for a specific antibodylike reagent interacting with antigen in the recipient as in the leukocyte donor. However, the accelerated rejection of test homografts applied 1 or 2 weeks after transfer suggests a more intimate participation of the recipient in this transaction.

In the transfer of skin homograft sensitivity the certainty that recipients have had no prior exposure to the antigens of the test system before transfer, coupled with the deliberate restriction to a single test with antigen at the time of and 1 or 2 weeks following transfer, make the two following conclusions valid:

TABLE 3

SUMMARY OF RESULTS OF TRANSFER OF SPECIFIC SENSITIVITY TO SKIN HOMOGRAFTS*

Mode of sensitization and time of collection of WBC from donor	Method of transfer	Material used for transfer	Results of transfer	
			No. of recipients	No. of positive transfers
First-set rejection	Systemic	Sensitive WBC extract	2	0
Second-set rejection	Systemic	Sensitive WBC extract	2	0
First- and second-set rejection	Local	Sensitive WBC extract	4	4
		Nonsensitive WBC extract	2	0
		Sensitive WBC extract on control graft	4	0
		Sensitive serum on test graft	3	0
Fourth-set rejection	Systemic	Sensitive WBC extract	6	6
11 Days after fourth-set rejection	Systemic	Sensitive WBC extract	4	0
5 Days after fourth-set "white-graft" application	Systemic	Sensitive WBC extract	4	0
		Sensitive plasma and RBC	1	0

* Reproduced from Lawrence *et al.*[30] with permission from the *Journal of Clinical Investigation.*

(1) Transfer factor is capable of endowing recipients with sensitivity *de novo*, excluding the possibility of recalling a dormant prior experience.

(2) Testing of recipients with antigen before and immediately after transfer is not an indispensable requirement for inaugurating the enduring effects observed.

The observation that DNase-treated leukocyte extracts are effective in the transfer of homograft sensitivity in man is in direct apposition to the experience in animal species where the use of viable cells has been mandatory.[8-11] This finding may be yet another reflection of the greater relative ease of sensitization and the greater intensity with which such sensitivity is expressed in humans, rather than a manifestation of any real species difference.[32]

In adapting the highly sensitive transfer system in human species to an evaluation of possible mechanisms of homograft rejection the biological properties of transfer factor have been extended. The use of leukocyte extracts allows the principles elucidated in relation to delayed hypersensitivity of bacterial[21,22] or fungal[33] origin to be transposed to a study of the mechanism of homograft hypersensitivity.[5,7,27,28] This type of experimental approach gives promise of delineating the antibody or antibodies concerned in homograft rejection, the effector cells involved and, perhaps, the tissue antigen(s) responsible for induction of homograft hypersensitivity.

References

1. Medawar, P. B. 1956–1957. The immunology of transplantation. Harvey Lectures. **52:** 144.
2. Medawar, P. B. 1959. Reactions to homologous tissue antigens in relation to hypersensitivity. *In* Cellular and Humoral Aspects of the Hypersensitive States : 504. H. S. Lawrence, Ed. Hoeber. New York, N. Y.
3. Brent, L. 1958. Tissue transplantation immunity. *In* Progress in Allergy. **5:** 271.
4. Gorer, P. A. 1956. Some recent work on tumor immunity. Advances in Cancer Research. **4:** 149.
5. Lawrence, H. S. 1959. Homograft sensitivity—an expression of the immunologic origins and consequences of individuality. Physiol. Rev. **39:** 811.
6. Medawar, P. B. 1954. General problems of immunity. *In* Preservation and Transplantation of Normal Tissues. Ciba Foundation Symposium. : 1. Little, Brown. Boston, Mass.
7. Lawrence, H. S. 1957. Similarities between homograft rejection and tuberculin-type allergy: a review of recent experimental findings. Ann. N. Y. Acad. Sci. **64**(**5**): 826.
8. Mitchison, N. A. 1954. Passive transfer of transplantation immunity. Proc. Roy. Soc. London **B142:** 72.
9. Billingham, R. E., L. Brent & P. B. Medawar. 1954. Quantitative studies on tissue transplantation immunity. II. The origin, strength and duration of actively and adoptively acquired immunity. Proc. Roy. Soc. London. **B143:** 58.
10. Billingham, R. E., L. Brent & P. B. Medawar. 1956. Quantitative studies on tissue transplantation immunity. III. Actively acquired tolerance. Phil. Trans. London. **B239:** 357.
11. Brent, L., J. Brown & P. B. Medawar. 1958. Skin transplantation immunity in relation to hypersensitivity. Lancet. **2:** 561.
12. Berrian, J. H. & L. Brent. 1958. Cell-bound antibodies in transplantation immunity. Ann. N. Y. Acad. Sci. **73**(3): 654.
13. Weaver, J. M., G. H. Algire, & R. T. Prehn. 1955. The growth of cells *in vivo* in diffusion chambers. II. The role of cells in the destruction of homografts in mice. J. Natl. Cancer Inst. **15:** 1737.
14. Stetson, C. A., Jr. & R. Demopoulos. 1958. Reactions of skin homografts with specific immune sera. Ann. N. Y. Acad. Sci. **73**(3): 687.
15. Stetson, C. A., Jr. 1959. The role of antibody in the rejection of homografts. *In* Mechanisms of Hypersensitivity. : 569. J. H. Shaffer, G. A. Lo Grippo & M. W. Chase, Eds. Little, Brown, Boston, Mass.
16. Good, R. A., R. L. Varco, J. B. Aust & S. J. Zak. 1957. Transplantation studies in patients with agammaglobulinemia. Ann. N. Y. Acad. Sci. **64**(5): 882.
17. Mitchison, N. A. 1955. Studies on the immunological response to foreign tumor transplants in the mouse. I. The role of lymph node cells in conferring immunity by adoptive transfer. J. Exptl. Med. **102:** 157.
18. Mitchison, N. A. & O. L. Dube. 1955. Studies on the immunologic response to foreign tumor transplants in the mouse. II. The relation between hemagglutinating antibody and graft resistance in normal mouse and mice pretreated with tissue preparations. J. Exptl. Med. **102:** 179.
19. Landsteiner, K. & M. W. Chase. 1940. Studies on the sensitization of animals with simple chemical compounds. VII. Skin sensitization by intraperitoneal injections. J. Exptl. Med. **71:** 237.
20. Chase, M. W. 1945. The cellular transfer of cutaneous hypersensitivity to tuberculin. Proc. Soc. Exptl. Biol. **59:** 134.
21. Lawrence, H. S. 1955. The transfer in humans of delayed skin sensitivity to strep-

tococcal M substance and to tuberculin with disrupted leucocytes. J. Clin. Invest. **34:** 219.
22. LAWRENCE, H. S. & A. M. PAPPENHEIMER, JR. 1956. The transfer of delayed hypersensitivity to diphtheria toxin in man. J. Exptl. Med. **104:** 321.
23. FREEDMAN, S. O., J. P. FISHER & R. A. COOKE. 1957. A study of leucocytic antibodies in allergic patients. J. Allergy. **28:** 501.
24. SANFORD, J. P. & J. A. BARNETT. Unpublished data.
25. MAURER, P. H. 1960. Production and transfer of delayed hypersensitivity to modified human serum proteins in humans. Federation Proc. **19:** 211.
26. GOOD, R. A. & S. J. ZAK. 1956. Disturbances in gamma globulin synthesis as "experiments of nature." Pediatrics. **18:** 109.
27. LAWRENCE, H. S. 1956. The delayed type of allergic inflammatory response. Am. J. Med. **20:** 428.
28. LAWRENCE, H. S. 1959. The transfer of hypersensitivity of the delayed type in man. *In* Cellular and Humoral Aspects of the Hypersensitive States. : 279. H. S. Lawrence, Ed. Hoeber. New York, N. Y.
29. PAPPENHEIMER, A. M., JR. 1956–1957. Hypersensitivity of the delayed type. Harvey Lectures. **52:** 100.
30. LAWRENCE, H. S., F. T. RAPAPORT, J. M. CONVERSE & W. S. TILLETT. 1960. Transfer of delayed hypersentivity to skin homografts with leucocyte extracts in man. J. Clin. Invest. **39:** 185.
31. CONVERSE, J. M. & F. T. RAPAPORT. 1956. The vascularization of skin autografts and homografts. An experimental study in man. Ann. Surg. **143:** 306.
32. LAWRENCE, H. S. 1959. Delayed hypersensitivity and the behaviour of the cellular transfer system in animal and man. *In* Mechanisms of Hypersensitivity. : 453. Little, Brown. Boston, Mass.
33. RAPAPORT, F. T., H. S. LAWRENCE, J. W. MILLAR, D. PAPPAGIANIS & C. E. SMITH. 1960. Transfer of delayed hypersensitivity to coccidioidin in man. J. Immunol. **84:** 358.

Discussion of the Paper

CHASE: One can have only encomiums for the beautiful demonstration that we see here of labor over a series of years devoted to a particular problem: that is, the problem of the mechanism of delayed-type sensitization.

What you have heard is presented almost in reverse order of history. The tables presented with the factual material derive from the transfer of tuberculin and streptococcal hypersensitivities and present data that were worked out, of course, by Lawrence before he turned his efforts to the study of the phenomenon of homograft rejection. The difficulties that he has now overcome have yielded to meticulous attention: how much of an allergenic stimulus to apply, when to harvest the cells, how long a period is afforded during which the cells remain capable of accomplishing the end desired. In the laboratory, one must solve such questions before one can make progress. Here we are privileged to see results of the adaptation of principles that have stemmed from concepts gained in the past.

This orderly progression of laboratory experience started with a system of cellular transfer that offered a lesser number of obstacles, in which it turned out that man was almost an elective species for such studies. The guinea pig, in which the phenomenon first came to light, has not lent itself to such facility in study of the mechanisms, and we are still unable to transfer hypersensitivity at will with extracts of guinea pig leukocytes.

Studies on the cellular transfer of delayed-type hypersensitivities in man offer elements of a conundrum. The area has not been elucidated to the extent that one would desire. On the one hand, it has proved easy to transfer bacterial allergies with peripheral leukocytes and, on the other, there has been considerable difficulty in the transfer of sensitivity to drugs: a person who remains dermally hypersensitive to a drug or chemical offers white cells that in only a few cases have been able to sensitize a negative man used as recipient. In the guinea pig, lymphocytes are harvested that—in the living state—transfer both sorts of delayed hypersensitivity equally well. What can be said of these "special differences"? In man, has one failed to learn the optimal time for taking cells for transfer in the two sorts of dermal hypersensitivity?

In the instance of homograft rejection by cellular transfer presently before us, it has been shown that as short a period as 11 days is sufficient to find the harvested cells no longer capable of functioning successfully in the transfer process. Using words that have been applied to the phenomenon, we may say that "transfer factor" was present at a particular time and only 11 days later was absent (in sufficient amount, at least). This finding raises a question of significance: if the transfer factor associated with tuberculin hypersensitivity (the area in which the chief studies of longevity of effect appear to have been made) is destined to persist for months, possibly years, in recipient individuals who, before cellular transfer, were nonreactors and thereafter permitted cellular transfer from first recipient to a second, and so on, what can be said of the "transfer factor" possessing such a short lifetime in cells that are able to transfer homograft rejection? The concept of "transfer factor" certainly deserves more study: it can hardly be regarded as other than a temporal statement to cover some of the information that has been gleaned.

PAUL MAURER: The only remarks I shall make have to do with confirmation of Lawrence's results on the efficacy of extracts of cells when transferred to humans to produce delayed allergy.

We have been working with an entirely different system, namely, a chemically modified human serum injected into humans. Individuals treated in this fashion respond when tested with the material with delayed reactions. When leukocytes from such sensitive donors were transferred to nonsensitive recipients the same type of delayed reactions were observed. These leukocytes could occur either in the form of viable or nonviable (frozen and thawed) extracts. The sera from these recipients who had been repeatedly tested with the material were studied by means of the chemical techniques available to us and also by use of many *in vivo* techniques or tests, such as the passive cutaneous anaphylaxis (PCA) test. In no case could we get positive tests for conventional serum antibody.

The main reason for making these remarks is that Lawrence, as you know, has experienced considerable difficulty in trying to convince people of the efficacy of these extracts. We, too, approached this problem with great skepticism; but these extracts now seem to us to be just as effective as Lawrence has always claimed.

PASSIVE TRANSFER OF ALLERGIC REACTIONS TO TUBERCULIN WITH PLASMA PROTEIN FRACTIONS FROM HYPERSENSITIVE GUINEA PIGS*

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Introduction

Mild forms of allergy of the so-called delayed type have been passively transferred from actively sensitized hosts to normal subjects with a variety of cell suspensions containing lymphocytes. The recent finding that extracts of human buffy coat leukocytes containing lymphocytes also will transfer these late-type reactions suggests that a factor derived from these cells is the active principle.[1] In contrast to the successes achieved with cells, passive transfer of late-type allergy has not been accomplished with the various methods of administering whole serum.[2] Admittedly, none of these serum transfer studies exposes recipient tissues to the physiological amounts of serum proteins that exist in the actively sensitized animal. That ineffective concentrations of active material or inhibitors present in whole serum may be the reason for these failures is suggested by a recent study in which plasma fractions separated from large volumes of blood were used passively to transfer late-type reactions to tuberculin.[3] In this study, plasma Fraction II (Fr. II) containing gamma globulin blocked transfer when the fractions were mixed. In some instances, large amounts of Fr. II passively transferred an Arthus type of reactivity to tuberculin. Since this original report, the transfer of Arthus-type sensitivity to tuberculin with gamma globulin has been confirmed by one group,[4] and the transfer of a late-type reaction to tuberculin with alpha globulin has been reconfirmed.[5] The report that follows describes further studies on the passive transfer of reactions to tuberculin with these plasma fractions.

Materials and Methods

Guinea pigs weighing more than 600 gm. were injected subcutaneously at weekly intervals with three 1.0-mg. doses of heat-killed human tubercle bacilli† in 0.1 ml. of mineral oil. Random booster doses (1.0 mg.) were given to maintain maximal tuberculin hypersensitivity. Selected animals, exhibiting tuberculin reactions of 10 mm. in diameter or greater and usually central necrosis when tested with 5 μg. purified protein derivative (PPD),‡ were bled from the heart 48 hours after skin testing. Pools containing 50 to 100 ml. of guinea pig plasma were fractionated according to the cold ethanol method previously described.[3]

Serum from each bleeding, samples from plasma pools, and aliquots from separate fractions were retained for electrophoretic analysis and total protein

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† Supplied by Parke, Davis & Company, Detroit, Mich.

‡ Supplied by Merck Sharpe & Dohme, Philadelphia, Pa.

determinations. Paper strip electrophoresis was done by the method of Jencks *et al.*,[6] with Whatman No. 1 filter paper strips. Scanning of strips was done with the Analytrol described by Durrum and Gilford,[7] with the B_3 cam. Immunoelectrophoresis was done by a modification of the method devised by Grabar and Williams.[8] Total proteins were determined by Nesslerization.[9]

Skin and eye reactions were observed grossly as described in the data that follow. Representative eye and skin biopsies were studied by standard pathological methods. The essential findings are described below. A more detailed description of the microscopic findings will be presented in a separate communication.

Normal guinea pigs weighing 300 to 500 gm. were used as recipients for passive transfer studies. In whole-body passive transfer experiments, fractions were administered intraperitoneally. The volume of the injection ranged between 1.0 and 10 ml. and contained between 10 and 60 mg. of protein. For local passive transfer experiments, fractions (0.2 ml.) were administered intracutaneously. Sites were identified with marking ink and tested at intervals thereafter. Skin tests in donors and recipients were done with 5 μg. PPD.

Replicate intraocular studies of separate normal recipient animals were done with fractions after anesthetizing the cornea with a drop of 1 per cent Pontocaine. A 30-gauge needle with a 0.25-ml. syringe was used to puncture the corneal limbus. Approximately 0.03 to 0.04 ml. of test fluid was injected into the far side of the anterior chamber. First- or second-strength PPD was then injected intracorneally. It is possible to determine precisely that the tuberculin is deposited intracorneally, since the material can be identified by a spreading zone of opalescence during injection. A third of the cornea was infiltrated with approximately 0.02 ml. PPD in saline. Control studies were done with fractions of normal plasma and with a human serum-antihuman serum (HS-AHS) and with a guinea pig serum-antiguinea pig serum (GPS-AGPS) rabbit antibody system. Actively sensitive guinea pigs also were tested with intracorneal PPD. Complicating secondary infection, deranged hydrodynamics, and progressive ophthalmia were avoided by adjusting the quantities of materials used to produce definite but limited corneal opacity in positive reactions. Insignificant reactions occurred in control studies. Complete recovery took place in the eyes recorded in the tables. Eyes removed for pathological study were of the type that normally recovered.

Results

In TABLE 1 are displayed composite data on the serum electrophoretic patterns of donor animals in a representative experiment. The hypergammaglobulinemia noted is similar to that reported elsewhere for guinea pigs sensitized by this method.[10] The yield of both Fr. II and Fr. IV-10 was greater in plasma pools from hypersensitive animals than it was in those from normal animals. It is not known, however, whether the increase in Fr. IV-10 in sensitive animals is due to a greater amount of alpha globulin, for immunoelectrophoretic studies of individual fractions indicate a considerable contamination of Fr. IV-10 with other serum proteins (FIGURES 1 to 3). In view of Seibert's finding that alpha globulins may contain polysaccharide that reacts with antisera of immunized animals,[11] Fr. II and Fr. IV-10 were injected intracutaneously into a group of highly sensitive, actively immunized guinea pigs. No immediate or delayed reactions were noted in any animals.

In TABLE 2 are displayed data on whole-body transfer studies with Fr. IV-10. Transfer studies were negative for 5 plasma pools from normal donors. Five transfers with plasma pools from sensitive donors gave positive results. Five other similar preparations of Fr. IV-10 gave borderline results. Two plasma pools from sensitive donors failed to transfer sensitivity to tuberculin. Reactions were of mild degree. They did not begin until 8 hours after PPD was placed in the skin and well after the initial injection site had subsided completely. Reactions consisted of erythema and induration. This was maximal at 24 hours. Larger reactions persisted for 48 hours; weaker reactions faded after the first day. The gross appearance of these reactions was similar to that of equally mild tuberculin reactions in less sensitive, actively immunized animals. Skin tests were repeated at intervals for a month in control and experimental animals. The only sensitivity encountered was that in positive transfer studies. This was lost in 2 to 3 weeks.

TABLE 1
ELECTROPHORETIC VALUES OF DONOR SERUM SAMPLES*

Serum	γ	β	α_2	α_1	Alb.	Total protein†
Hypersensitive (18)						
I, 15.6; N, 8.1						
(%)	18.2	15.8	21.8	4.1	40.1	
(gm./100 ml.)‡	1.1	0.9	1.3	0.2	2.3	5.7
Normal (13)						
(%)	5.4	14.7	19.5	2.5	57.8	
(gm./100 ml.)	0.3	0.9	1.2	0.2	3.5	6.1

* Mean values.

† Total protein is expressed as grams per 100 ml.

‡ I, diameter of induration in millimeters; N, diameter of necrosis in millimeters.

Microscopic sections of active and passive reaction sites of the same intensity revealed hyperemia and a mixed cell response. In general, passive transfer reactions seemed to have fewer cells than did active reactions of similar intensity. At 24 hours, polymorphonuclear leukocytes (PMN), eosinophils, and a variety of mononuclear cells were seen in groups in the lumen of blood vessels, around dilated vessels, and scattered diffusely through the layers of the skin. At 48 hours, fewer PMN and eosinophils were present. At 5 to 6 days, a sparse mononuclear cell reaction was seen.

TABLE 3 gives data on the local passive transfer of reactions to tuberculin with Fr. IV-10. A late-appearing skin reaction to PPD was transferred locally with 3 of 4 preparations of Fr. IV-10 and with 4 preparations of Fr. II. Three other preparations of Fr. II transferred borderline reactions. Two preparations of Fr. II from sensitive donors failed to transfer tuberculin sensitivity. Gross and microscopic reactions were like those of equal intensity in actively sensitized animals.

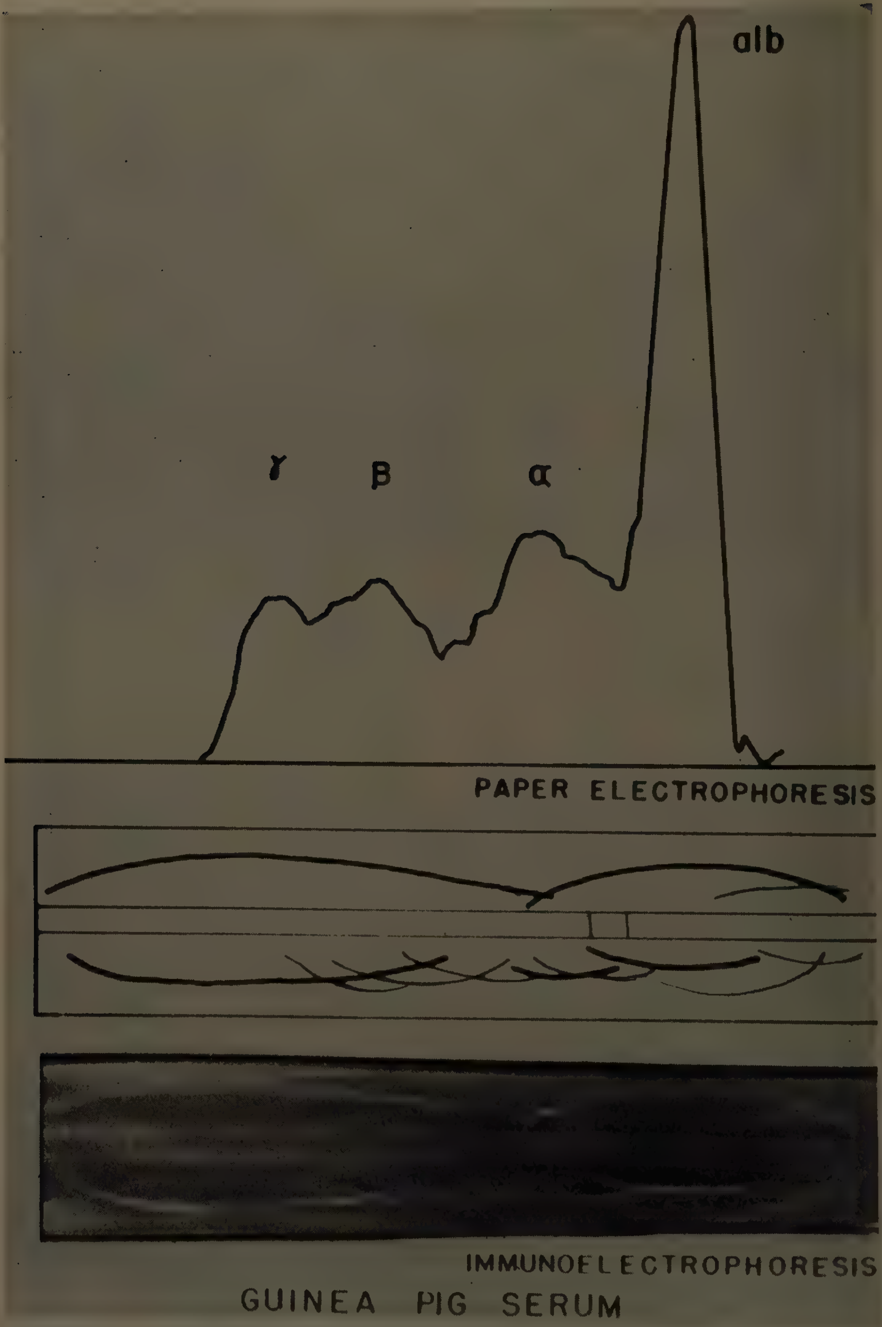

FIGURE 1. Paper strip and immunoelectrophoretic analysis of representative guinea pig serum from a tuberculin-sensitive donor for preparation of plasma fractions for transfer experiments.

In TABLES 4 and 5 are shown data on passive transfer of ocular reactions to tuberculin. A late-appearing reaction to tuberculin was transferred to the cornea with 6 preparations of Fr. IV-10. Two preparations of Fr. IV-10 transferred borderline reactions, and 2 failed to transfer sensitivity to tuberculin. Ten of 10 preparations of Fr. II from sensitive donors transferred sensitivity to tuberculin. Fr. II from normal donors did not transfer signifi-

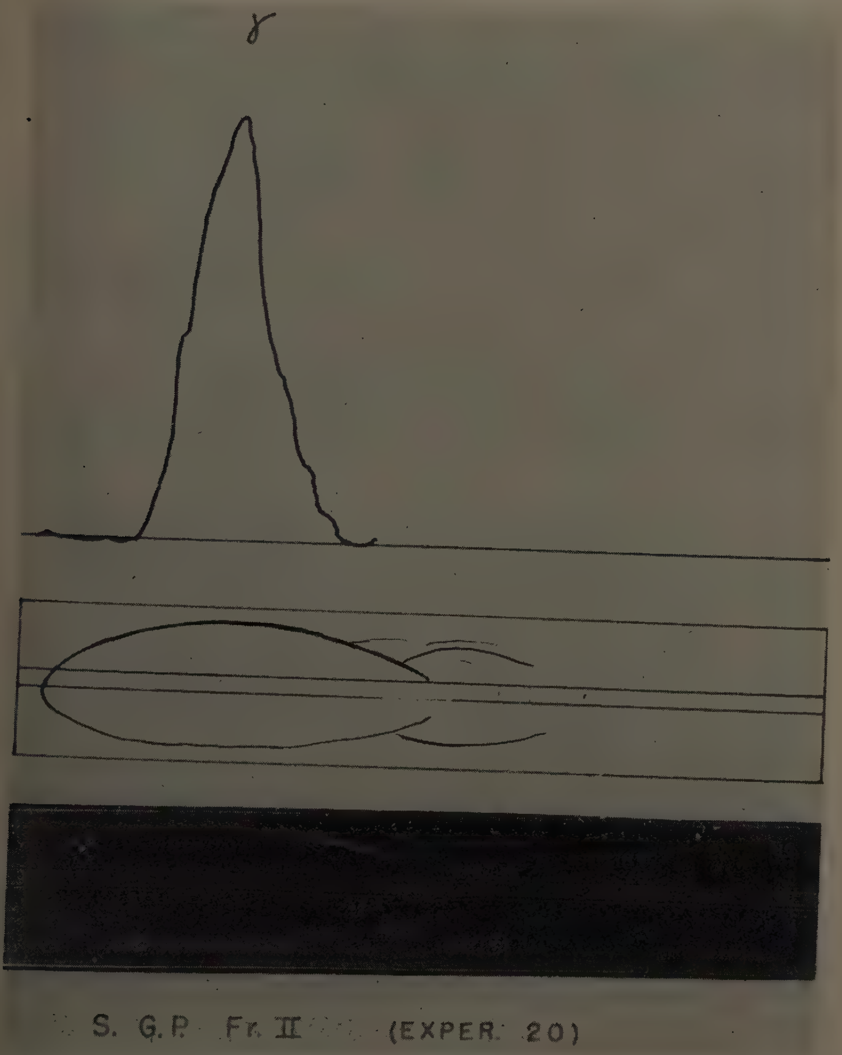

FIGURE 2. Qualitative paper-strip and immunoelectrophoretic analysis of a representative sample of Fraction II from tuberculin-sensitive donors. The schematic diagram of the gel-diffusion study indicates that the gamma-globulin is contaminated with small amounts of alpha-globulin. For purposes of these methods the protein concentrations were adjusted to obtain comparable qualitative information. The height of peaks does not reflect the quantitative value of individual proteins present in fractions. S. G.P., sensitive guinea pig.

cant sensitivity to tuberculin. Corneal clouding was graded on a scale of 0 to ++++, the higher value indicating opacity obscuring the iris and lens. Opalescence following intracorneal injections in control animals disappeared within 4 hours. In experimental animals, increasing opacity began within 12 hours. Reactions reached their peaks in 24 hours. Stronger reactions persisted after this time. Both active and passive reactions and those produced with the HS-AHS system (TABLE 6) underwent a similar sequence of grossly visible changes. By 5 to 6 days, eyes appeared normal.

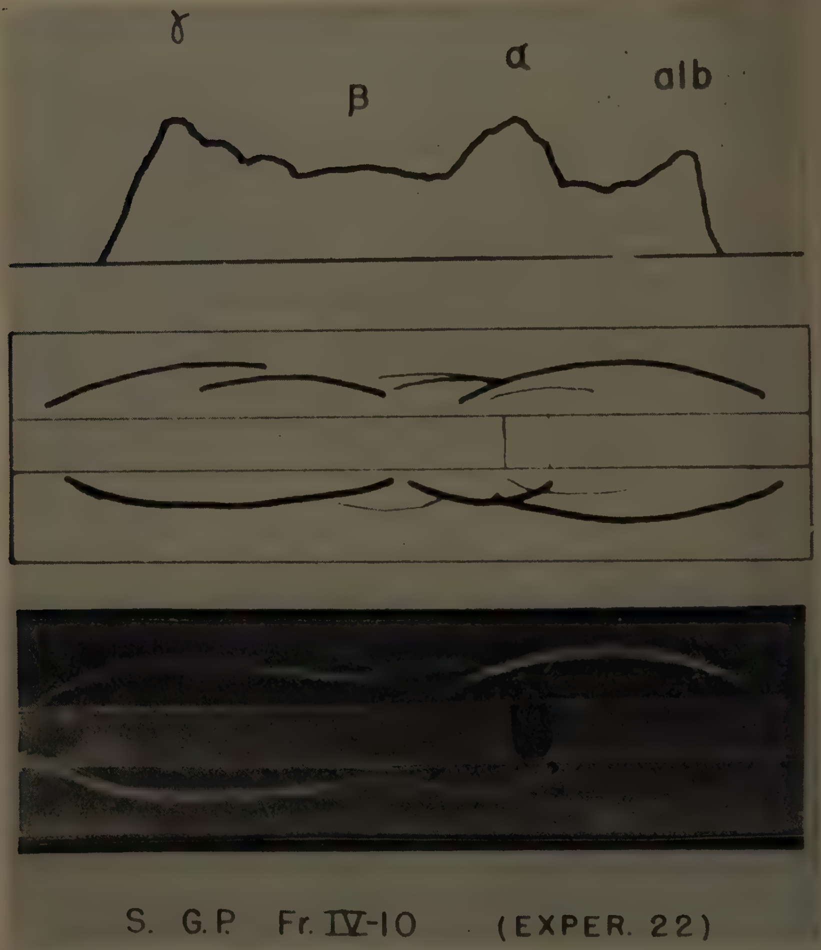

FIGURE 3. Qualitative paper-strip and immunolelectrophoretic analysis donors. The schematic diagram of the gel-diffusion study indicates that alpha globulin is contaminated with many other plasma proteins. The height of peaks in these qualitative studies does not reflect quantitative yields of the proteins present in fractions. S. G.P., sensitive guinea pig.

Microscopic study (TABLE 7) of representative reactions may be summarized

TABLE 2

PASSIVE TRANSFER OF TUBERCULIN SENSITIVITY BY INTRAPERITONEAL INJECTION OF FRACTION IV-10

Expt.	Plasma vol. (ml.)	Donor sensitivity* (48 hours)		Recipient sensitivity*				
				24 hours		48 hours		Day test
		I	N	I	N	I	N	
Normal								
2	90	0		0				6
				0				13
3	98	0		0				6
				1				13
4	115	0		0				6
				0				13
6	120	0		0		1		6
				2				13
24	90	0		2				4
				2				7
				0				12
				2				18
Sensitive								
1	50	15	7	9	2	7	1	5
				0				22
7	75	14.5	7	0				6
				5		1		13
8	135	15	1	5		2		6
				0				13
15	50	14.2	7.6	†				1
				0				7
				3				10
				0				17
17	108	13.5	5.9	0				5
				0				17
				7		2		20
				7		1		23
				2†				30
18	108	15.8	7.0	0				6
				8		6		11
				8		6		14
				6		1		17
				5†				24
				5				31
19	95	16.7	7.3	5		4		2
				3‡				9

* I, diameter of induration in millimeters; N, diameter of necrosis in millimeters.
† Positive corneal tuberculin test.
‡ Negative corneal tuberculin test.

Table 2—(Continued)

Expt.	Plasma vol. (ml.)	Donor sensitivity* (48 hours)		Recipient sensitivity*				
				24 hours		48 hours		Day test
		I	N	I	N	I	N	
19 (cont.)				0				16
				2				19
				3				23
				0				26
				0				30
20	110	17.7	8.1	4				7
				5				10
				5				15
				4				18
				4		5		20
				5		4		22
				4				26
				0				28
21	100	20.9	8.6	0†				1
				8		5		6
				0				14
				0				16
				2				21
				0				23
				1				27
22§	112	17.6	7.1	2				6
				0				8
				2				13
				0				15
				0				19
				2				28
23	85	18.6	8.1	0				2
				0				6
				0				9
				0				13
				5				21
				2				27
25	80	17.0	6.1	1				4
				5				12
				3				18
				1				26

* I, diameter of induration in millimeters; N, diameter of necrosis in millimeters.

† Positive corneal tuberculin test.

‡ Negative corneal tuberculin test.

§ Only one third of total Fraction IV-10 recovered was used here for passive transfer.

by saying that both active sensitivity reactions and passively transferred reactions due to Fr. II and Fr. IV-10 were similar. These reactions were characterized by hyperemia of vessels at the limbus of the cornea and by mixed cellular response in the ciliary body, limbus, and cornea. At 24 hours PMN, eosinophils, and a variety of mononuclear cells appeared in vessels, in perivascular spaces, and between the corneal fibers. By 48 hours PMN and eosinophils were less noticeable and, by 5 to 6 days, only a few mononuclear cells remained in the limbus. The initial reactions caused by HS-AHS appeared at 4 hours and were accompanied by a more intense PMN and eosinophilic response than was seen with the other reactions. When these reactions subsided they contained a few mononuclear cells. Similar but less intense changes were seen in passive reactions produced with the GPS-AGPS system. Opacity

TABLE 3

LOCAL PASSIVE TRANSFER OF REACTIVITY TO TUBERCULIN IN THE SKIN BY MEANS OF PLASMA FRACTIONS*

Fraction IV-10			Fraction II		
Expt.	Interval†	Reactivity at 24 hours	Expt.	Interval	Reactivity at 24 hours
Normal					
24	3 hour	−	24	3 hour	−−−
	24 hour	−		24 hour	−−−
Sensitive					
21	1 hour	++	16§	15 min.	−−
				24 hour	−−
22	15 min.	++	18§	15 min.	−−
				3 hour	±
				24 hour	−−−
23	15 min.	−−	19	15 min.	−−
	24 hour	++		3 hour	−
				24 hour	++−
25‡	3 hour	±	20	15 min.	−−
	24 hour	±		3 hour	+
				24 hour	−+−
			21	1 hour	+
				6 hour	++
				24 hour	−
			22	1 hour	−
				6 hour	−+
				24 hour	−
			23	1 hour	−
				6 hour	−+
				24 hour	−
			25	3 hour	−−
				24 hour	+−
			26	3 hour	−−−
				24 hour	+±+

* Reactions consisted primarily of erythema and an ill-defined edema greater in the fraction-plus-PPD than in control sites of fraction-plus-saline in place of PPD and initial PPD retested with saline. All PPD control sites were negative.

† Interval between intracutaneous injection of 0.2 ml. fraction and 5 μg. PPD.

‡ Whole-body transfer was borderline in expt. 25.

§ Fractions from expt. 16 and 18 were held over one month as a precipitate at −5° C. before use.

TABLE 4

PASSIVE TRANSFER OF TUBERCULIN SENSITIVITY TO THE CORNEA OF NORMAL GUINEA PIGS BY THE INJECTION OF FRACTION IV-10 INTO THE ANTERIOR CHAMBER*

Expt.	Plasma vol. (ml.)	Donor sensitivity†		Recipient sensitivity			
		I	N	.02 µg. PPD		5 µg. PPD	
				24 hours	48 hours	24 hours	48 hours
Normal 10	105	0		++ + +	0 + +		
11	100	0		± ± + ±	0 + ‡ +		
24	90	0		± 0	+ 0	+ +	± ±
Sensitive 8	47	13.2	8.2	++++ ++	++++ ++		
9	135	14.5	1	++ ++	+ +		
13	103	14.7	6.6	+ + + +	++ + ‡ +		
14	80	16.5	7.3	++ ++ ++ +++ +++	0 ‡ ++ ‡ ++		
20	110	17.7	8.1	++ +++ ++	0 ++ 0	++++ +++ ++++	++++ ++ ++++
21	100	20.9	8.6	++ ++ ±	± 0 0	+++ + ++ ++++	++ 0 ++ ++
22	112	17.6	7.1	0 + + + +	± ++ + + +	+ ++++ +	+ ++ 0

* Iritis was graded by an observer unaware of the injection code. Different times of bleeding, degrees of donor hypersensitivity, and variation in the amount of fraction leaking from the anterior chamber immediately after injection are factors that account for variations in the severity of iritis in sensitive Fraction IV-10 experiments. Substitution of saline for either fraction or PPD resulted in at most a corneal opacity of +.

† I, diameter of induration in millimeters; N, diameter of necrosis in millimeters.

‡ Eye removed for pathological examination.

Table 4—(Continued)

Expt.	Plasma vol. (ml.)	Donor sensitivity†		Recipient sensitivity			
				.02 µg. PPD		5 µg. PPD	
		I	N	24 hours	48 hours	24 hours	48 hours
23	85	18.6	8.1	++ +++	++ +	++ +++ +	± ± 0
25	80	17.0	6.1	± ±	± +	+ +	± +
26	102	15.4	5.5	++ +++ +++	+++ +++ +++	+ ++++ +++	+ +++ ++

of the cornea produced by tuberculin in actively sensitive animals was accompanied by more cellular infiltration than was seen in passively transferred reactions of similar intensity.

Comment

In this report, the transfer of reactions to tuberculin by plasma fractions is described. Whole-body transfers have been accomplished with Fr. IV-10 obtained from 50 to 100 ml. of plasma collected from actively sensitized guinea pigs which were skin-tested 48 hours before bleeding. Both whole-body and local-transfer cutaneous reactions to tuberculin were characterized by erythema and induration of mild degree. Reactions developed after 8 hours and were maximal at 24 hours. More intense reactions persisted for 48 hours. Sensitivity appeared within 24 hours in local transfers and in 1 to 2 weeks in whole-body transfers. Local reactivity waned within a few days and was gone in a week. Reactivity in whole-body transfers usually lasted 2 to 3 weeks.

Microscopic study of representative, passively transferred skin reactions revealed a mild response that was similar in quality to, but less cellular than, equally mild reactions induced in actively sensitized animals. At 12 hours PMN, eosinophils, and a variety of mononuclear cells were present. These cells appeared in the lumen of capillaries, in clusters adjacent to blood vessels, and scattered throughout the layers of the skin. At 24 hours fewer PMN and eosinophils were seen. At 48 hours mononuclear cells were predominant in the subsiding reactions. These changes will be reported in detail elsewhere. Except for the relative mildness of these reactions and perhaps a greater proportion of PMN in the early stages, they were essentially the same as those described by Gell and Hinde[12] in rabbits, for the evolution of tuberculin reactions uncomplicated by necrosis or complex secondary changes.

The passive transfer studies with the eyes as the local site of deposit of Fr. II and Fr. IV-10 from sensitive donors in general paralleled the findings in

TABLE 5

PASSIVE TRANSFER OF TUBERCULIN SENSITIVITY TO THE CORNEA OF NORMAL GUINEA PIGS BY THE INJECTION OF FRACTION II INTO THE ANTERIOR CHAMBER*

Expt.	Plasma vol. (ml.)	Donor sensitivity†		Recipient sensitivity			
		I	N	.02 µg. PPD		5 µg. PPD	
				24 hours	48 hours	24 hours	48 hours
Normal							
3	98	0		+ ++ +	0 + +		
6	120	0		+ +	0 0		
11	100	0		+ + +	0 0 ‡		
24	90	0		± ±	+ ±	++ + +	± ± ±
Sensitive							
5	75	15.1	7.2	+++ ++++ +++ +++ +++	+++ ++++ +++ +++ ++		
7	75	14.5	7	++++ +++	+++ ++		
12	103	14.7	6.6	++++ ++ ++++ +++	++ 0 +++ ++		
13	103	17.9	4.9	++ ++ +++ ++ ++ ++ ++	± ++ ‡ 0 ++ ++ ++		
16	115	18	6.1	++++ ++	++++ +	++ ++	++ +++
20	110	17.7	8.1	++ +	++ +	++ ++++	++ ++++

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* The same grading scheme was used here as in TABLE 4.

† I, diameter of induration in millimeters; N, diameter of necrosis in millimeters.

‡ Eye removed for pathological study.

TABLE 5—(Continued)

Expt.	Plasma vol. (ml.)	Donor sensitivity†		Recipient sensitivity			
				.02 µg. PPD		5 µg. PPD	
		I	N	24 hours	48 hours	24 hours	48 hours
21	100	20.9	8.6	+++ + ++	++‡ + ++	++‡ ++	++
23	85	18.6	8.1	++++ ++	+++ ++		
25	80	17.0	6.1	++ +++ ±	+ +++ +	++++ +++	++++ +++
26	102	15.4	5.5	++ + +++	++ + ++	++ +++ +	++ + ++

skin studies. Active and passive corneal sensitivity to tuberculin underwent a similar gross and microscopic evolution. The one surprising finding was that the reactivity caused by Fr. II was similar to that caused by Fr. IV-10 and to that seen in actively sensitive animals. Since neither fraction is entirely free of the other, it is difficult to interpret these findings further than to say that transfers with fractions were more like active tuberculin hypersensitivity than they were like passively transferred reactions produced by a known antigen-antibody system.

Perhaps the major implication of these transfer studies is the emphasis they place on separating and concentrating humoral factors for the analysis of their roles in hypersensitivity reactions. In whole-body transfers a single animal receives approximately the amount of Fr. IV-10 to be found in 4 to 5 times his own plasma volume. Ordinarily, this amount of serum cannot be given to an animal at one time. In these experiments the removal of albumin from plasma made it possible to do so without serious disturbances in the recipient's blood volume. In local transfers the fraction used was equal to approximately 2 to 3 ml. of plasma. In the eye transfer experiments the amount of fraction injected was derived from the equivalent of 0.5 ml. of plasma. It is likely that these amounts approach the order of magnitude of local interstitial fluid turnover at reaction sites in the course of mild late-type hypersensitivity reactions. Despite the volume of plasma used, fractions probably contained little active material. This fact and the absence of a specific hypersensitive capacity on the part of the recipient cells that were seen to infiltrate these reactions no doubt are reasons for the mild degree of cellular response in these reactions. In this respect, these transfers with plasma fractions differ from transfers of tuberculin hypersensitivity that have been accomplished with living cells or with cell extracts. They also differ from reactions to tuberculin that occur in the actively sensitized host, where complex changes not readily separated by morphologic studies go to make up the so-called delayed-type hypersensitivity.

TABLE 6

IRITIS PRODUCED BY HUMAN AND GUINEA PIG SERUM ANTIGEN AND RABBIT ANTISERUM IN NORMAL GUINEA PIGS*

Injection site†		Reaction		
Anterior chamber	Cornea	24 hours	48 hours	72 hours
AHS	HS	++++	++++	+++
		++++	++++	++++
		++++	++++	++++
		+++	‡	
		++	+++	‡
		++	++	++
		++	++	+
		+++	++	+
		++	++	+
		++	+++	++
		++	±	0
1:10 AHS	HS	++++	++++	++++
1:100 AHS	HS	+++	+++	+++
		++	++	±
		++	+	±
1:1000 AHS	HS	+	+	+
AGPS	GPS	++	++	+‡
		++	+	+
		++	+	+
		++	++	+
		++	+	±
		+++	++‡	
		+++‡		
		++	+	±

* The same grading scheme was used here as in TABLE 4. Controls with saline in the anterior chamber and antigen in the cornea, and the reverse situation, with antisera in the anterior chamber and saline in the cornea, were at most + at 24 hours only. Controls with normal rabbit serum in the anterior chamber and saline in the cornea were the same. However, in several instances whole serum in the cornea and also in the anterior chamber produced an opacity (++) that persisted for 24 hours, but disappeared by 48 hours.

† AHS, antihuman serum; HS, human serum; AGPS, antiguinea pig serum; GPS, guinea pig serum.

‡ Eye removed for pathological study.

Summary

Whole-body, local cutaneous, and ocular sensitivity to tuberculin has been passively transferred from actively sensitized to normal guinea pigs. This has been accomplished with intraperitoneal, intracutaneous, and intraocular injections, respectively, of plasma Fr. IV-10 obtained from large plasma pools by cold ethanol fractionation. Fr. II from sensitive animals, when given intraocularly, also has been used for passive transfer of sensitivity to tuberculin.

Immunoelectrophoretic study of these plasma fractions indicates that Fr. II consists largely of gamma globulin qualitatively contaminated with alpha globulin. Fr. IV-10 consists of alpha globulin contaminated with other plasma proteins.

TABLE 7

MICROSCOPIC OBSERVATIONS ON ACTIVE AND PASSIVE OCULAR REACTIONS TO TUBERCULIN AND PASSIVE OCULAR REACTIONS TO ANTIGEN-ANTIBODY SYSTEMS

Chamber	Cornea	Time (hours)	Corneal opacity	Microscopic changes			
				Limbus			Cells in cornea
				PMN	Eos	Mono	
Sensitive guinea pigs							
—	PPD	12	++	++	+	++	+++
—	PPD	24	+++	++	+	++	+++
—	PPD	48	+++	+	±	++	+++
Normal guinea pigs							
N IV-10	PPD	24	+	+	+	±	±
N IV-10	PPD	48	0	±	±	0	±
S IV-10	PPD	24	+++	++	+	++	++
S IV-10	PPD	48	++	±	±	++	+
S IV-10	PPD	3 days	+	0	0	±	0
S IV-10	PPD	6 days	0	0	0	±	0
N II	PPD	24	+	±	±	±	+
S II	PPD	24	+++	+	+	±	++
S II	PPD	48	+++	±	±	+	+
AHS	HS	4	±	++	++	0	±
AHS	HS	24	+++	+++	+++	+	+++
AHS	HS	48	+++	++	+	+	++
AHS	HS	5 days	0	0	0	±	0
AGPS	GPS	24	+++	++	++	+	+++
AGPS	GPS	48	++	+	+	++	+

* Corneal opacity graded as in TABLE 4. Microscopic changes: cell grading done on a scale of 0 to ++++.

PMN, polymorphonuclear leukocytes; Eos, eosinophils; Mono, mononucleocytes; HS, human serum; AHS, antihuman serum rabbit antibody; GPS guinea pig serum; AGPS, antiguinea pig serum rabbit antibody; S, fraction derived from plasma of sensitized guinea pigs; N, fraction derived from plasma of normal guinea pigs.

The transferred cutaneous and ocular reactions were characterized by a late-appearing mild inflammation maximal at 24 hours. Passive hypersensitivity waned after several days. Microscopic study of skin and eye reactions showed changes similar to but not identical with mild tuberculin reactions in actively sensitized control animals.

References

1. LAWRENCE, H. S. 1955. The transfer of hypersensitivity of the delayed type in man. *In* Cellular and Humoral Aspects of Hypersensitivity States. : 279. Hoeber. New York, N. Y.

2. Chase, M. W. 1945. The cellular transfer of cutaneous hypersensitivity to tuberculin. Proc. Soc. Exptl. Biol. Med. **59:** 134.
3. Cole, L. R. & C. B. Favour. 1955. Correlations between plasma protein fractions, antibody titers, and the passive transfer of delayed and immediate cutaneous reactivity to tuberculin, PPD and tuberculopolysaccharides. J. Exptl. Med. **101:** 391.
4. Ehrenkrantz, N. J. & B. H. Waksman. 1956. Failure to transfer tuberculin sensitivity passively with plasma fractions containing alpha globulin. J. Exptl. Med. **104:** 935.
5. Cole, L. R., R. L. Paldino, R. W. Henderson & A. F. West. 1959. Passive transfer of a delayed-type skin reactivity to tuberculin PPD. Am. Rev. Resp. Dis. **80:** 398.
6. Jencks, W. P., E. L. Durrum & M. R. Jetton. 1955. Paper electrophoresis as a quantitative method: serum proteins. Biochem. J. **60:** 205.
7. Durrum, E. L. & S. R. Gilford. 1955. Recording integrating photoelectric and radioactive scanner for paper electrophoresis and chromatography. Rev. Sci. Instruments. **26:** 51.
8. Grabar, P. & C. A. Williams, Jr. 1955. Méthode immuno-électrophorétique d'analyse de mélange de substances antigéniques. Biochim. et Biophys. Acta. **17:** 67.
9. Lanni, F., M. L. Dillon & J. W. Beard. 1950. Determination of nitrogen in serological precipitates and their biological materials. Proc. Soc. Exptl. Biol. Med. **74:** 4.
10. Favour, C. B. 1958. Relation between serum gamma globulin levels and hypersensitivity induced by vaccination with tubercle bacilli. Clin. Research. **6:** 103.
11. Seibert, F. B., M. L. Pfaff & M. V. Seibert. 1948. A serum polysaccharide in tuberculosis and carcinoma. Arch. Biochem. **18:** 279.
12. Gell, P. G. H. & I. T. Hinde. 1951. The histology of the tuberculin reaction and its modification by cortisone. Brit. J. Exptl. Pathol. **32:** 516.

Discussion of the Paper

CHASE: To hold the concept that some protein factor capable of conveying a delayed-type sensitization can circulate in the serum and induce allergic conversion of previously negative individuals upon transfer requires, of course, bold thinking. Large quantities of serum have been transferred by many workers without positive result. Favour has made transfers by effecting, as it were, a concentration of the serum proteins, from four to six or more animals, so that the equivalent of much whole serum can be conveyed in small volume. Furthermore, it is possible that exclusion of some protein fractions may be an advantage, but this last needs further investigation. I think that Favour is to be congratulated in persisting in this area, and I am sure that we have not yet heard the last of the story.

Let us consider briefly the facts now presented. In the earlier experiments of Cole and Favour whole serum seemed not to permit transfer but, after the proteins of the serum had been subjected to fractionation, one of these was reported able to effect transfer, namely, IV-10.

The animals from which the serum had been drawn, and in the present experiments again have been drawn, were sensitized with very large amounts of dead tubercle bacilli; we know that the serum of such animals is laden richly with antituberculocarbohydrate, possesses small amounts only of antituberculoprotein, and has a certain quota of antibody that is directed probably against a complex of polysaccharide with a short chain of amino acids (the latter component being insufficient to cause it to precipitate when saturated ammonium sulfate is applied).

We have, then, a very complex mixture of antibodies present in this starting material. It is known that tuberculin is also complex: PPD is "pure" in name only. There is carbohydrate in it, as well as more than one protein. In this complex system, the transfer experiments have been conceived and executed boldly, searching for the "needle in the haystack." This is selected from pools of the serum provided by quite a few animals, each of which is sensitive in its own right, and the product finally isolated from these large pools is sufficient when transferred to an animal now and then to confer a type of reactivity.

I was privileged to see Favour's manuscript in advance; the comment must be offered that the skin reaction given by only a single individual among the recipient animals seemed to me to be of possible significance. The reactions achieved in the eye are of another order and, with these, I lack experience to offer comments, but Russell Weiser may wish to add to this particular story.

As Favour has very carefully pointed out, there are certain differences between what we conceive of as the delayed type of reaction to tuberculin and some of the results that he has obtained. For example, after cellular transfer, or after the transfer of cellular extracts in man, whole series of repeated positive tests become possible.

It might be suggested, among future things to be done, that Favour might choose to try a rather purified tuberculocarbohydrate instead of PPD to elicit the reactions in animals injected with the serum fractions that were used; again, in my opinion, a proper control might be the use of the corresponding fraction of a pool of guinea pig antibody (not rabbit antibody) that has arisen, not from animals that possess delayed-type allergy, but from guinea pigs that have been stimulated only with proteins (in such fashion that they possess only antiprotein); recipient animals might be tested with the antigenic protein.

It is necessary to keep in mind the fact that the total amount of protein that Favour has been able to introduce into the eye remains rather small, owing to limitation of volume and irritative concentrations of foreign materials; also, that very many of the protein molecules introduced will consist of inert protein. Such a state of affairs should encourage the possibility of further selective concentration. Eventually the fractions now being studied will, I am sure, be compared for possible capacity to effect lympholysis, and all of these other facets will be explored further.

R. S. WEISER: I am particularly impressed with the eye reactions. We have always regarded the corneal reaction as a much more dependable measure of tuberculin hypersensitivity than the skin reaction.

I think that one could add that whenever one gets corneal reactions, they really represent strong reactions, in so far as tuberculin hypersensitivity is concerned.

Indeed, to get a good corneal reaction in a tuberculous guinea pig, the animal must be very sensitive. In other words, the skin reaction must be correspondingly very high.

C. B. FAVOUR: The only comment I desire to add is that the tuberculin reaction, as we know it, is probably a very complex phenomenon, and that what we have been dealing with in these fractions, or with anything the fractions may contain, is probably only a very small part of what is occurring.

This I tried to indicate in the difference in the cellular responses that take place. It may well be that cells do more than one thing when they transfer tuberculin reactivity: that perhaps there is a double function of the cells that Lawrence takes apart and puts back in, one of which is something like a serum factor and the other, something else that accounts for the very long-lasting effects that he sees.

The type of thing we are dealing with apparently is of a short effect very similar to the short effect one finds in passive transfer with almost any serum component of the anaphylactic sort, where the serum has a half life and finally decays and is lost.

Another point of some interest is that, in the active tuberculin reaction, the cellular infiltration that takes place probably represents some form of a chemotactic phenomenon in which circulating cells (active cells capable themselves of transferring tuberculin reactivity) are concentrated locally; thus you have within the host and at the site of the reaction a so-called local passive transfer from the host itself.

When we have tried to transfer this reaction passively with protein fractions, we are not obtaining help from the host in that respect, because none of the host cells actually has any of this capacity, and it would be unlikely that the same cellular response, or even the same morphology, would occur.

Chase is quite right; these reactions do not look like the classic tuberculin reaction, and I think he is making a strong point in saying that this would not be called the typical "positive-tuberculin reaction" that one speaks about by other means of transfer.

HUMORAL ASPECTS OF THE IMMUNE RESPONSE TO HOMOGRAFTS*

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Various aspects of the immune response to homografts have been discussed in recent years by Gorer,[1,2] Kaliss,[3] Hauschka,[4] Snell,[5,6] Lawrence,[7] Medawar,[8,9] and Brent.[10] In each of these reviews will be found a treatment of the general subject of the circulating antibodies found in homograft recipients. There seems now to be general agreement that circulating antibodies of one sort or another have been found in association with the reaction to homologous tissue transplants under so many circumstances that there remains no doubt that they are a characteristic feature of the immune response involved. During the past few years some attention has been paid to the apparently diverse nature of the antibodies formed and to the possible role of each of these in the various phenomena of transplantation biology. At least four different kinds of antibody activity have been reported in isoimmune sera: erythrocyte agglutination, leukocyte agglutination, a protective or tumor-neutralizing effect, and a cytotoxic effect. While each of these effects may be mediated by a separate and distinct antibody species, the erythrocyte-agglutinating and leukocyte agglutinating activities of these sera may be due to one antibody, and the protective and cytotoxic effects to another. Alternatively, it may be that a single antibody is responsible for all of these effects. It is to be hoped that absorption and fractionation studies will result in better characterization of these antibodies and of the antigenic determinants with which they react.

Without any doubt, the antibodies that have received the most study are the murine hemagglutinins. The original demonstration by Gorer[11] of a humoral antibody response to homografts involved this system, and the availability of numerous inbred lines and transplantable tumors in this species has made the mouse the favorite experimental animal for studies on transplantation immunology. The hemagglutinins in this species are sometimes demonstrable by direct agglutination of donor erythrocytes in saline, but more usually are demonstrated by the elegant "Dextran" method of Gorer and Mikulska.[12] These antibodies are sometimes unstable, and high titered sera may show troublesome prozones, but the methods for their detection[12,13] are sensitive and reliable and have provided means for the careful analysis of the kinetics of formation[13,14] and duration of persistence[14,15] of these antibodies and of the genetic determination of the antigens against which they are directed. It is clear that these antigens are determined by the same genetic units that determine histocompatibility. Gorer *et al.*[13] have shown that hemagglutinins are demonstrable in the serum some days before the onset of any visible manifestations of the homograft reaction, and Mitchison and Dube[14] found that the hemagglutinin response to second-set grafts was brisk and resembled a classic

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"secondary" immunological response. While much of the work done with these antibodies has involved the use of transplantable leukemias, solid neoplasms, and ascites tumors, entirely similar primary and secondary hemagglutinin responses have been recorded[16] after skin grafting in mice.

The experiments of Mitchison and Dube[14] and of Hildemann and Medawar[17] would seem to indicate that these hemagglutinins are not involved in the breakdown of homografts, since they may be absent under some circumstances in which transplantation immunity exists and may be present in high titer under conditions in which transplantation immunity is absent. It has been suggested that these antibodies, far from working the destruction of homografts, may actually be involved in the phenomena of "enhancement" and that, if these antibodies have any effect on homografts, it is to prolong their survival.[1-10]

The leukocyte agglutinins have not been as extensively studied, although good methods[18,19] for their demonstration have been developed. In Amos' experiments,[18] leukocyte agglutinin titers ran roughly parallel to the hemagglutinin titers, both in genetic experiments and in absorption experiments, and it may well be that the same antibody is responsible for both effects.

For many years, isoimmune sera have been known to protect against transmissible neoplasms of various kinds. The extensive literature dealing with this phenomenon has been well reviewed by Gorer.[1] In general, the conditions of successful experiments seem to be the production of a hyperimmune serum and the demonstration of its protective effect by mixing serum and tumor cells prior to inoculation or by injecting the serum at about the same time that the tumor is implanted. In contrast to the encouraging results obtained in such experiments, attempts at influencing the growth of *established* tumors by injections of serum have been almost uniformly unsuccessful. In an effort to account for these paradoxical findings, Amos[15] showed that a transplantable neoplasm was able to absorb antibodies *in vivo* provided the antibodies were administered at the same time as the tumor transplant; but when the tumor was first given eighteen hours to establish itself, the injection of antiserum was not followed by any detectable absorption *in vivo*, as though the tumor antigens were inaccessible to the antibodies, which declined in titer with the expected half life.

Relatively little is known about the relationship between these "protective" antibodies and the agglutinins. Absorption experiments[20] show that the protective activity of a serum is not necessarily dependent on hemagglutinating antibody, and that nearly all of the hemagglutinating activity is in the gamma globulin fraction of mouse isoimmune serum, while much of the protective antibody appears to be associated with the alpha and beta globulins. The nature of the antigens against which the protective antibodies are directed is also not yet entirely clear. Some but not all of these antibodies appear to be directed against the H_2 antigens.[1,20] It cannot be stated with certainty that the experiments of Harris *et al.*[21] and of Billingham and Sparrow[22] indicate the existence of protective antibodies effective against normal cells, but this interpretation seems most likely.

Historically, the most recent isoantibodies to be described are the "cytotoxic" antibodies, so called because they produce damage and lysis of intact cells *in* *vitro* in the presence of complement. Kalfayan and Kidd[23] described damage to Brown-Pearce carcinoma cells produced by isoimmune rabbit serum and complement, and Schreck and Preston[24] later described a similar phenomenon. Gorer and O'Gorman[25] devised a method for the detection of cytotoxic isoantibody in mouse serum based on the counting of damaged cells whose nuclei had taken up eosin Y or trypan blue. They found that normal as well as neoplastic homologous cells were damaged by specific antibody and complement. In genetic experiments, the cytotoxic antibodies also appeared to be directed against the major histocompatibility antigens.[13] It seems quite likely that these antibodies are the ones involved in the protection or neutralization phenomena described above.

In view of the possible significance of these antibodies in the mediation of homograft immunity, it is rather surprising that no systematic study of the qualitative and quantitative aspects of their formation has been reported. In their classic study of the passive transfer of homograft immunity, Mitchison and Dube[14] examined their sera only for hemagglutinins and by a procedure that may have been not entirely adequate.[1] In a more recent study of the antibody response to homologous cells and fractions thereof, Hildemann and Medawar[17] used both an established hemagglutination technique and a hemolytic technique to measure antibodies reacting with donor erythrocytes, but did not examine their sera for leukoagglutinins or for cytotoxic activity. Reports of failures to achieve passive transfer of homograft immunity with serum have in no instance given account of the spectrum of antibody activities in the test sera. Except in the case of the murine hemagglutinins,[13-15] little or nothing is known of the time of appearance and persistence of the various antibodies, of the relative effectiveness of various immunization procedures, and of dose-response relationships. A systematic study of these problems has been commenced in this laboratory, and the present report is concerned with some of the findings with respect to cytotoxic antibodies.

Materials and Methods

Mice of the BALB/c and C57BL lines were obtained from the Jackson Memorial Laboratories. Immunization of BALB/c mice against C57BL spleen cells (living or lyophilized) was accomplished by the injection of the material in a total volume of 0.1 ml. per mouse divided between the 4 footpads. In some experiments the cell or lyophilized tissue suspensions were incorporated into Freund's adjuvant (to the "incomplete" adjuvant obtained from Difco Laboratories was added 5-mg./ml. killed, dried human tubercle bacilli) by the mechanical emulsification of equal parts of the saline tissue suspension and the adjuvant-tubercle bacillus suspension. Booster injections were given after various intervals as indicated. Mice were bled from the retroorbital venous plexus, and the sera of several mice were pooled for testing. Cytotoxic antibody titers were determined by the following modification of the method of Gorer and O'Gorman.[25] The target cells were usually those of the ascites tumor E.L.4, kindly supplied by Bernard Amos, Roswell Park Memorial Institute, Buffalo, N. Y. and maintained by weekly passage in C57BL mice. After harvesting, the cells were washed once and suspended to a suitable concentration in balanced salt solution containing glucose. For the test, 0.2 ml. of this cell suspension was added to 0.2 ml. of serum dilution, and after 15 to 30 min. incubation in a water bath at 37° C. with constant shaking, 0.1 ml. guinea pig complement was added to each tube. After 20 to 30 min. further incubation with shaking, 1.0 ml. of 0.1 per cent eosin Y in balanced salt solution was added and mixed, and a drop of the suspension was transferred to a glass slide, ringed with vaseline, and covered with a cover slip. The number of stained cells observed under the microscope was expressed either as a percentage of the total or, more usually, as + (0–25 per cent), ++ (26–50 per cent), +++ (51–75 per cent) or ++++ (76–100 per cent). The end points in serum titration were reproducible and sharp, and the serum titers were expressed as the last dilution of serum giving ++ (50 per cent) or more staining. Skin grafts were performed by removing 10 × 20 mm. full-thickness skin samples from the dorsum of the thorax, inverting the graft and scraping away the subcutaneous tissue, and sewing the graft into place with silk sutures. Graft recipients were kept in individual cages for subsequent observation, and dressings were found to be unnecessary.

Experimental Results

The demonstration of cytotoxic antibodies in isoimmune sera proved simple, and the technique described permitted the determination of serum titers that proved reproducible to within one dilution. The morphologic alterations produced in the E.L.4 cells are illustrated in FIGURE 1, and resemble those described by Howard Green and B. D. Goldberg elsewhere in this volume. In practice, the staining of the cells[25] provides a more convenient index of damage in preparations where some, but not all, of the cells have been affected.

Results illustrating the main directions of our experiments will be seen in TABLES 1 to 4. They show that it is possible to produce high titers of cytotoxic antibodies by the injection of live spleen tissue followed by a number of booster injections and that this response is considerably enhanced by the use of Freund's whole adjuvant (TABLES 1 and 2). TABLE 2 shows that no cytotoxic antibodies were detected after repeated injections of lyophilized tissue, but that lyophilized tissue in adjuvant gave rise to unexpectedly high titers of these antibodies. TABLE 4 shows the localization of these antibodies within serum protein fractions.

In the past two years, evidence has accumulated to support the hypothesis that antibodies of this type may be responsible for the rejection of at least some types of homografts *in vivo*. Early reports[26-29] of the results of experiments with diffusion chambers indicated that homologous cells were not destroyed in immunized hosts unless host cells had access to the chamber. These findings have been interpreted as evidence against the mediation of homograft immunity by circulating antibodies.[1,7-10] More recently, however, Amos and Wakefield[30] and Algire[31] have shown that various tumors growing in cell-impenetrable chambers are destroyed rapidly, in fact, in immunized hosts. Algire[31] suggests that earlier failures to observe this effect may have been due to inadequate immunization of the hosts. In any event, the more recent evidence available from diffusion chamber experiments suggests that cytotoxic antibody may be necessary and sufficient to mediate homograft destruction.

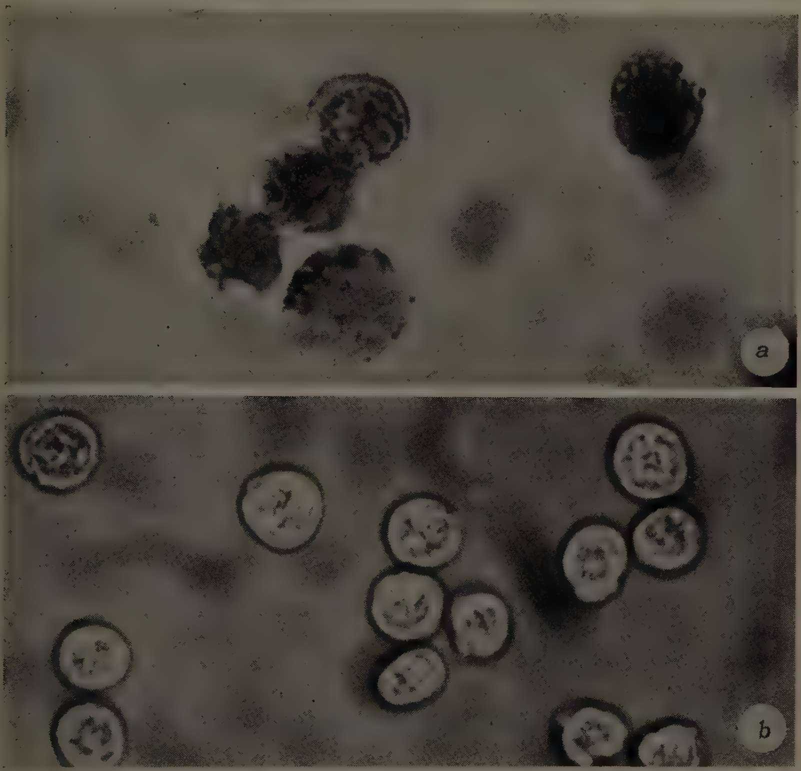

Figure 1. Photomicrograph of (*a*) E.L.4 cells in balanced salt solution to which had been added a 1/100 dilution of a BALB/c anti-C57BL serum and guinea pig complement (the cells are swollen and have lost much of their cytoplasmic contents to the medium); (*b*) normal E.L.4 cells in balanced salt solution to which had been added antiserum but no complement. Eosin Y had been added to both preparations, and the cells in *a*, but not in *b*, show staining.

Table 1
Cytotoxic Antibody Titers of Antisera Produced in BALB/c Mice Immunized with C57BL Spleen Cells or E.L.4 Tumor

Day	Antisera produced against		
	Intact spleen cells	Intact spleen cells + adj.	E.L.4 cells
0	0	0	0
22	5	125	25
31	125	625	

Immunizing injections of approximately 10^7 cells were given on day 0 and booster injections were given on days 8 and 24. In this and similar experiments the cytotoxic antibody titer was determined against E.L.4 cell suspensions. Higher titers of cytotoxic antibody were found regularly after immunization with cell-adjuvant mixtures than after injection of homologous cells alone, and appreciable increases in titer were found after booster injections.

In some preliminary experiments, we have injected antisera of high cytotoxic activity intravenously into BALB/c mice bearing established C57BL skin homografts in order to see whether antibodies capable of killing C57BL cells *in vitro* could exert a similar effect *in vivo*. In no case has the injection of such serum, given 2 or 4 days after grafting, produced any visible effects on the test graft. It will be recalled, however, that Amos' experiments[15] indicate that established grafts are inaccessible to circulating antibodies. It seemed possible that an established skin graft might be destroyed if steps were taken to increase the permeability of the graft vessels, allowing cytotoxic antibody in the blood

TABLE 2

CYTOTOXIC ANTIBODY RESPONSE OF BALB/c MICE IMMUNIZED AGAINST INTACT C57BL SPLEEN CELLS AND LYOPHILIZED C57BL SPLEEN CELLS

Day	Antisera produced against		
	Intact spleen cells	Lyophilized spleen	Lyophilized spleen-adj.
0	0	0	0
8	100	0	0
17	5	0	25
55	25	0	5
62	625	25	625
68	625	125	3125
76	100	20	500

Each mouse was immunized against approximately 10^7 cells on day 0, and similar injections were given as boosters on days 8 and 17. All booster injections on days 55 and 62 contained Freund's adjuvant. No booster injections were given on day 68. An early but transient cytotoxic antibody response is seen in the animals receiving intact spleen cells, while lyophilized spleen cells in equivalent amount stimulated cytotoxic antibody formation only when incorporated into Freund's adjuvant. Booster injections with adjuvant resulted in marked increases in titer in all groups. The antibody titers were determined against E.L.4 cells, and are expressed as the reciprocal of the highest dilution of serum showing cytotoxicity.

TABLE 3

COMPARISON OF THE CYTOTOXIC ACTIVITY OF BALB/c ANTI-E.L.4 SERUM FOR E.L.4 CELLS AND FOR NORMAL C57BL LYMPHOCYTES

	Cytotoxic activity at serum dilution					
	1/50	1/100	1/200	1/400	1/800	BSS
E.L.4	++++	++++	++++	+++	0	0
Lymphocytes	++++	+++	++	+	+	+
E.L.4	–	–	82%	35%	1%	1%
Lymphocytes	–	–	31%	13%	8%	9%

Some of the cells in lymph-node cell suspensions showed staining in balanced salt solution in the absence of antibody, perhaps due to nonspecific damage in the preparation of the cell suspensions. In both titrations, the numbers of E.L.4 cells and of lymphocytes were approximately the same. In the first titration, there appears to be a 1- or 2-tube difference in the titer against the two types of cells, the lymphocytes being more resistant to the cytotoxic antibody-complement system. In the second titration, the actual percentages of stained cells are shown, and a similar result is evident.

stream to penetrate vessel walls and make effective contact with graft antigens. Benacerraf *et al.*[32] showed that small intradermal doses of histamine lead to the fixation or accumulation of circulating antibody at the site of injection, and we have found in further experiments that the injection of small amounts of histamine into an established skin graft results in prompt local hemorrhage and necrosis of the graft in the presence of circulating cytotoxic antibody.

FIGURE 2 illustrates the reaction observed. Application of xylene or brombenzene to the skin also leads to a transient local increase in vascular permeability,[33] probably due to local histamine release; when this was done to established skin grafts in animals that had previously received intravenous injections of cytotoxic antiserum, graft breakdown occurred in the painted areas.

TABLE 4

LOCALIZATION OF CYTOTOXIC ANTIBODY ACTIVITY IN FRACTIONS OF BALB/c ANTI-C57BL SERUM PREPARED BY ELECTROPHORESIS AND BY ANALYTIC ULTRACENTRIFUGATION

Fractionation procedure	Tubes	Fraction	Cytotoxicity
Starch block electrophoresis	1–3	γ-globulin	0
	4–6	γ-globulin	++++
	7–9	γ-globulin	+
	10–12	β-globulin	0
	13–15	α-globulin	0
	16–20	albumin	0
Analytic ultracentrifuge	1	19S γ-glob.	0
	2	19S γ-glob.	0
	3		0
	4	7S γ-glob.	0
	5	7S γ-glob.	++
	6	7S γ-glob.	++++
	7	7S γ-glob.	0
	8		0

All of the cytotoxic activity of this serum was found in the mid-region of the gamma globulin fraction as obtained by starch block electrophoresis, and sedimented as a 7S gamma globulin in the ultracentrifuge.

These preliminary experiments suggest that in established homografts there exists a "blood-graft barrier" that more or less isolates the graft from any circulating antigraft antibodies. Breakdown of this barrier may be required in order for antibody to make effective contact with graft antigen. In the normal homograft reaction, increased permeability of graft vessels may result from a local delayed hypersensitivity reaction of the type recently demonstrated by Brent *et al.*[34] The result of a local breakdown in this hypothetical "blood-graft barrier" would amount to a local injection of antibody-containing serum, a procedure already reported to produce breakdown of established homografts.[35] Now that it is possible to produce antisera of known high cytotoxic activity, it should be possible to test this hypothesis in various ways, and perhaps to reconcile some of the conflicting evidence implicating delayed hypersensitivity and humoral antibodies in homograft reactions.

In summary, the existence of cytotoxic activity in isoimmune serum[25] has

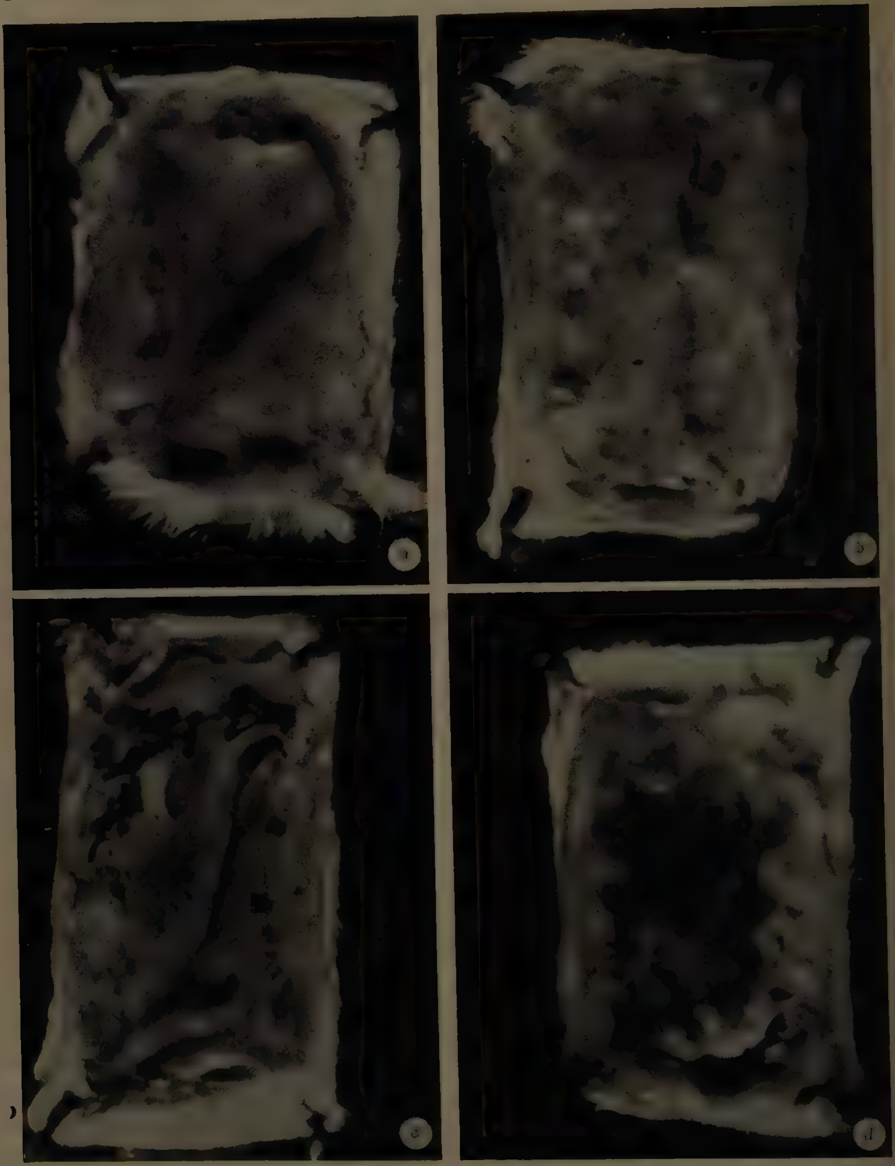

FIGURE 2. Four C57Bl skin grafts removed (with some of the surrounding host skin) from BALB/c mice on the sixth day after grafting. Breakdown of grafts of this size (200 mm^2.) in this strain combination is usually not evident until the tenth day. On the fourth day, the mice bearing grafts *c* and *d* received intravenous injections of 0.3 ml. of BALB/c anti-C57BL serum with a cytotoxic antibody titer of 1/625. After 1 day, the grafts had suffered no visible damage. At that time, injections of histamine (25γ) were made into the centers of grafts *b* and *d*. During the next 24 hours, the hemorrhagic necrosis vislbe in graft *d* developed. Histologic examination of these grafts showed complete destruction of the graft *d* in the region of the histamine injection, while graft *b* showed only the slight extravasation of erythrocytes to be expected after injection of this dose of histamine, the epidermis and dermis showing no necrosis or inflammatory change. The grafts have been inverted and photographed to show the hemorrhagic necrosis in the dermis of graft *d*.

been confirmed and, with the use of Freund's adjuvants and suitable booster injections, it has been found possible to raise antisera with cytotoxic activities considerably higher than previously described. The finding that lyophilized tissue is also effective in the production of cytotoxic antibodies is of considerable interest, since such preparations are generally considered to be devoid or nearly devoid of transplantation antigens.[3,6,10] It will be of interest to compare the hemagglutinating, leukocyte agglutinating, and cytotoxic activities of these sera. Such studies, now in progress, should form a useful background for the more adequate evaluation of the role of antibodies in homograft immunity.

Acknowledgment

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References

1. Gorer, P. A. 1956. Advances in Cancer Research. **4:** 149.
2. Gorer, P. A. 1956. Ann. N. Y. Acad. Sci. **63**(5): 882.
3. Kaliss, N. 1958. Cancer Research. **18:** 992.
4. Hauschka, T. S. 1952. Cancer Research. **12:** 615.
5. Snell, G. D. 1957. Cancer Research. **17:** 2.
6. Snell, G. D. 1957. Ann. Rev. Microbiol. **11:** 439.
7. Lawrence, H. S. 1959. Physiol. Rev. **39:** 811.
8. Medawar, P. B. 1956–1957. Harvey Lectures. **52:** 144.
9. Medawar, P. B. 1959. *In* Cellular and Humoral Aspects of the Hypersensitive States: 504. H. S. Lawrence, Ed. Hoeber. New York, N. Y.
10. Brent, L. 1958. Progr. in Allergy **5:** 271.
11. Gorer, P. A. 1937. J. Pathol. Bacteriol. **44:** 691.
12. Gorer, P. A. & Z. B. Mikulska. 1954. Cancer Research. **14:** 651.
13. Gorer, P. A., Z. B. Mikulska & P. O'Gorman. 1959. Immunology. **2:** 211.
14. Mitchison, N. A. & O. L. Dube. 1955. J. Exptl. Med. **102:** 179.
15. Amos, D. B. 1955. Brit. J. Cancer. **9:** 216.
16. Amos, D. B., P. A. Gorer, Z. B. Mikulska, R. E. Billingham & E. M. Sparrow. 1954. Brit. J. Exptl. Pathol. **35:** 203.
17. Hildemann, W. H. & P. B. Medawar. 1959. Immunology. **2:** 44.
18. Amos, D. B. 1953. Brit. J. Exptl. Pathol. **34:** 464.
19. Terasaki, P. I., J. A. Cannon & W. P. Longmire. 1959. Proc. Soc. Exptl. Biol. Med. **102:** 280.
20. Amos, D. B. & E. D. Day. 1957. Ann. N. Y. Acad. Sci. **64**(5): 851.
21. Harris, S., T. N. Harris & M. Farber. 1958. J. Exptl. Med. **108:** 411.
22. Billingham, R. E. & E. N. Sparrow. 1954. J. Exptl. Biol. **31:** 16.
23. Kalfayan, B. & J. G. Kidd. 1953. J. Exptl. Med. **97:** 145.
24. Schreck, R. & F. W. Preston. 1956. J. Natl. Cancer Inst. **16:** 1021.
25. Gorer, P. A. & P. O'Gorman. 1956. Transplantation Bull. **3:** 142.
26. Algire, G. H. 1954. J. Natl. Cancer. Inst. **15:** 403.
27. Algire, G. H. 1957. Ann. N. Y. Acad. Sci. **69**(4): 663.
28. Algire, G. H., J. M. Weaver & R. T. Prehn. 1957. Ann. N. Y. Acad. Sci. **64**(5): 1009.
29. Weaver, J. M. G. H. Algire & R. T. Prehn. 1955. J. Natl. Cancer. Inst. **15:** 1737.
30. Amos, D. B. & J. D. Wakefield. 1958. J. Natl. Cancer Inst. **21:** 657.
31. Algire, G. H. 1959. J. Natl. Cancer Inst. **23:** 435.
32. Benacerraf, B., G. Biozzi & B. N. Halpern. 1954. J. Immunol. **73:** 318.
33. Thomas, L. & C. A. Stetson. 1948. Proc. Soc. Exptl. Biol. Med. **69:** 409.
34. Brent, L., J. Brown & P. B. Medawar. 1958. Lancet: 561.
35. Stetson, C. A. & R. Demopoulos. 1958. Ann. N. Y. Acad. Sci. **73**(3): 687.

ANTIBODY RESPONSE TO HOMOGRAFTS: V. CYTOTOXIC EFFECTS UPON LYMPHOCYTES AS MEASURED BY TIME-LAPSE CINEMATOGRAPHY*

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Introduction

In vitro cytotoxic effects of sera from animals previously injected with homologous tumor cells have been reported by many workers in recent years.[5,6,10,13,14] These antibodies have also been shown to react upon normal lymphoid cells by Gorer and O'Gorman,[7] Gorer and Boyse,[8] and Hellström[9]. Following immunization with normal homologous tissues, *in vitro* cytotoxic effects have been difficult to demonstrate.[1,11]

The present work demonstrates the presence of cytotoxic "antibodies" in the sera of animals injected with pooled splenic cells that act *in vitro* upon homologous lymphocytes. Time-lapse cinematography was employed to determine the viability of cells. Motility was taken as the criterion of viability. These motion pictures have been effective also in the study of cytological changes taking place in lymphocytes killed by homologous "immune" sera.

Methods

Immunization. New Hampshire (NH) and White Leghorn (WL) chickens were injected reciprocally with pooled splenic tissue. The splenic cells were teased out with forceps in Hanks' balanced salt solution and injected subcutaneously. Spleens from a total of 20 to 40 chickens were injected 2 to 3 times as indicated in TABLE 2. With such large doses of splenic cells, a substantial mortality occurred in the injected birds—presumably due to the graft against host reaction. Marked loss of weight and atrophy of the thymus and of the spleen were the most notable symptoms, although systematic autopsies were done. Of 44 injected birds, 22 died in 2 to 4 weeks after the last injection.

Test for cytotoxicity. Blood from immunized animals was taken 1 to 4 hours previous to each test, using 1 ml. of chick embryo extract per 10 ml. of blood to facilitate clotting. The sera obtained were used either undiluted or diluted 1:2 with Hanks' balanced salt solution. No external source of complement was added. Lymphocytes obtained from the blood by methods previously described[15] were taken from birds of the opposite breed to the serum being tested and added to the sera in Rose tissue culture chambers[12] using 800,000 to 1,200,000 lymphocytes per chamber; penicillin (100 U./ml.) and streptomycin (20 γ/ml.) were also added in some cases. At various times after incubation at 37° C., time-lapse motion pictures were taken of the cultures.

Cinematography and analysis of film. An inverted phase-contrast microscope with ×25 or ×40 objective was employed to photograph lymphocytes from the bottom of the culture chambers. Representative fields of culture were photographed for about 15 to 35 min. at 16 frames per minute. By repeated

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forward and reverse projection of the film, the number of motile and nonmotile lymphocytes was counted. From this, the percentages of the motile cells were calculated and tabulated in TABLES 1 and 2.

TABLE 1

THE EFFECT OF SERA FROM ANIMALS INJECTED WITH HOMOLOGOUS SPLENIC TISSUE ON THE MOTILITY OF HOMOLOGOUS LYMPHOCYTES

Immunization*	No. days between last inj. and bleeding	Serum No.	Lymphocytes No.	Time of incubation (hours)	No. of motile cells/ Total No. counted	Per cent motile	Mean percentage motile in normal serum for incubation period (Tab. 2)	Decrease in motility from normal (%)
10, 33d, *10*	13	3	156	5	2/85	2.3	84.3 ± 6.2	97.3
10, 33d, *10*	15	3	642	8½	78/119	65.5	79.4 ± 9.1	17.5
10, 33d, *10*	15	3	642	27½	34/105	32.4	39.8 ± 23.6	18.6
10, 33d, *10*	13	429	156	6	1/65	1.5	84.3 ± 6.2	98.2
10, 33d, *10*	15	429	643	9½	74/96	77.0	79.4 ± 9.1	3.0
10, 33d, *10*	15	429	643	28½	2/59	3.4	39.8 ± 23.6	91.5
10, 33d, *10*	15	429	642	10½	0/87	0.0	79.4 ± 9.1	100.0
10, 33d, *10*	17	429	642	3	1/26	3.8	84.3 ± 6.2	95.5
20, 51d, *10*, 100d, *10*	9	427	690	5	10/134	7.4	84.3 ± 6.2	91.2
20, 51d, *10*, 100d, *10*	9	427	690	7	4/181	2.2	84.3 ± 6.2	97.4
20, 51d, *10*, 100d, *10*	23	427	686	2	35/120	29.2	84.3 ± 6.2	65.4
20, 134d, *11*, 49d, *10*	12	397	690	8	16/170	9.4	84.3 ± 6.2	88.8
20, 134d, *11*, 49d, *10*	12	397	690	22	1/120	0.8	39.8 ± 23.6	98.0
20, 134d, *11*, 49d, *10*	6	397	586	4½	79/100	79.0	84.3 ± 6.2	6.3
20, 134d, *11*, 49d, *10*	6	397	586	24	0/81	0.0	39.8 ± 23.6	100.0
11, 49d, *10*	6	435	586	3	186/198	93.9	84.3 ± 6.2	−11.4
11, 49d, *10*	6	435	586	22	29/108	26.9	39.8 ± 23.6	32.4
10, 183d, *10*	6	283	586	1½	134/143	93.7	84.3 ± 6.2	−11.2
10, 183d, *10*	6	283	586	20	19/69	27.5	39.8 ± 23.6	30.9
20, 51d, *10*	67	427	602	15½	113/160	70.6	79.4 ± 9.1	11.1
20, 51d, *10*	67	427	602	27½	38/267	14.2	39.8 ± 23.6	64.3
20, 51d, *10*	67	427	547	16½	184/252	73.0	79.4 ± 9.1	8.6
20, 51d, *10*	67	427	547	28½	10/140	7.1	39.8 ± 23.6	82.2
20, 51d, *10*	67	427	314	17½	260/316	82.3	79.4 ± 9.1	−3.7
20, 51d, *10*	67	427	314	29½	104/182	57.1	39.8 ± 23.6	−43.5

* Number of donors in splenic pool in italics, and number of days between injections.

Results

Due to the limited time lymphocytes are normally viable in tissue cultures, only short-term cultures up to 30 hours were attempted. TABLE 1 shows the percentage of motile cells after various intervals of incubation with normal sera. It is evident that up to 18 hours of incubation most cells remain viable. The mean percentage viable at 1 to 8 hours was 84.3 ± 6.2 per cent and 79.4 ±

9.1 per cent at 8½ to 18 hours. Chambers examined after 21 to 30 hours of incubation showed a notable decrease in the proportion of living cells (39.8 ± 23.6 per cent), with a wider spread of variability between different tests.

TABLE 1 shows the effect of "immune" sera upon homologous lymphocytes. All tests were performed concurrently with normal serum controls (TABLE 2) using the same lymphocyte suspensions. Sera from immunized animals have a marked effect upon lymphocytes. In many instances this effect can be seen in as short an incubation period as 3 hours. In other instances apparently a longer interval up to 30 hours is needed to demonstrate toxicity. Relatively short terms of incubation or the long period between immunization and testing of the serum (67 days) probably can account for the high proportions of viable cells found in some instances after incubation with immune serum.

TABLE 2

THE VIABILITY OF LYMPHOCYTES IN NORMAL HOMOLOGOUS SERA AS MEASURED BY COUNTS FROM TIME-LAPSE CINEMATOGRAPHS

Time of incubation (hours)	Serum no.	Lymphocytes no.	No. motile/total no. counted	Per cent motile
5½	628	156	73/116	62.9
6½	625	156	131/153	85.6
3	615	383	120/130	92.3
2	686	586	187/195	95.9
3½	685	586	124/136	91.2
5½	554	586	112/140	80.0
7½	693	690	139/168	82.7
5	586	690	103/123	83.7
				Mean per cent 84.3 ± 6.2
16	568	602	145/189	76.7
17	568	547	194/218	89.0
9	639	642	182/217	83.8
10	640	643	109/160	68.1
				Mean per cent 79.4 ± 9.1
28	568	602	66/145	45.5
30	568	314	190/237	80.2
28	639	642	30/116	25.8
29	640	643	34/67	50.7
21	686	586	77/168	45.8
23	685	586	58/250	23.2
25	554	586	6/78	7.7
				Mean per cent 39.8 ± 23.6

As a means of keeping lymphocytes viable longer in tissue culture, lowered temperatures of incubation were tried. Incubation at 24° C. for 20 hours and 37° C. for 6 to 9 hours resulted in viability with different normal sera of 93.6, 82.3, 82.2, and 83.3 per cent. Incubation for 8 hours at 37° C., 23 hours at room temperature, and 7½ hours at 37° C. resulted in cultures with 63.6 and 84.9 per cent motility. Also of some interest is 1 trial in which immune serum plus lymphocytes resulted in 29.2 per cent viability after 2 hours of culture. This same serum tested with lymphocytes from the same donor the next day had apparently lost much of its cytotoxic effect, 95.6 per cent of the lymphocytes still being viable after 2 hours' incubation.

From analysis of time-lapse motion pictures taken of lymphocytes incubated in homologous immune sera, the following sequence of events has been observed: The normal motility of lymphocytes with the formation of lobes, as shown in FIGURE 1, gradually ceases, but the cell continues to "attempt" to move in various directions as shown by the rapid movement of the outer margin of the cell in all directions. This small movement without the protrusion of lobes can best be seen at higher magnifications. Perhaps due to the adherence of lymphocytes to the glass caused by immune serum, the cell remains stationary. Sometimes long filaments have been seen to appear at this stage (FIGURE 2). These filaments may be identical to the myelin forms shown by Bessis to develop from the surface of injured cells (particularly from red blood cells, but also from white cells).[2] Then, at periods varying from minutes to hours, all movement ceases. The nuclear volume gradually decreases with a simultaneous swelling

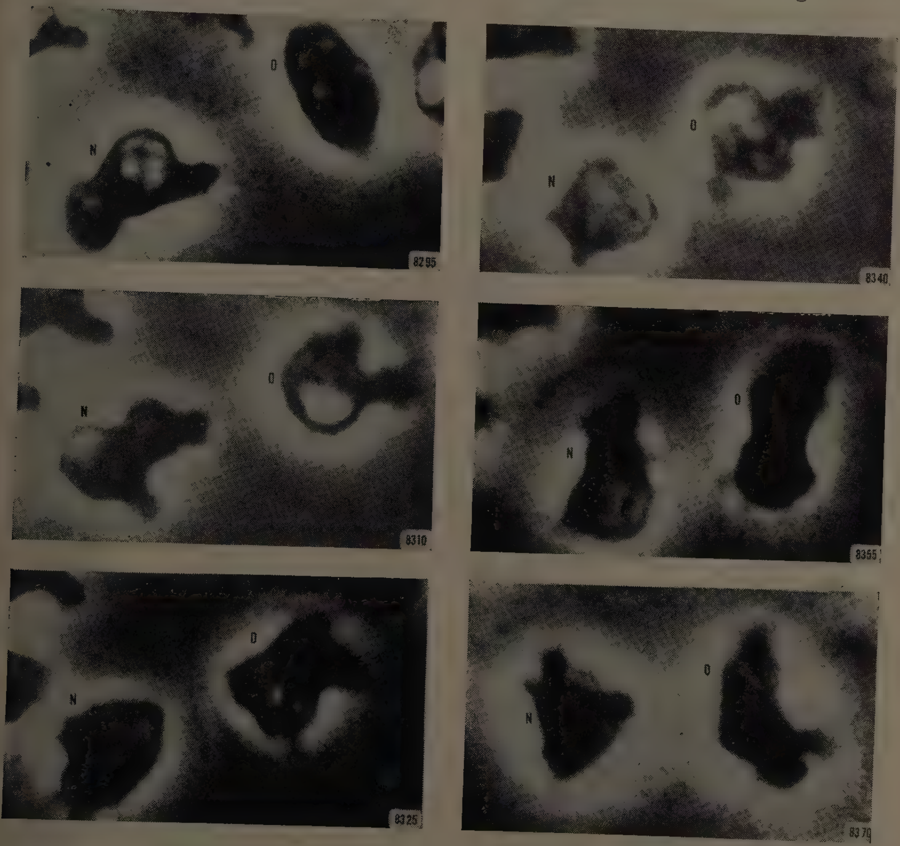

FIGURE 1. Lymphocytes in normal serum can be seen to move about actively. The numbers indicate the number of seconds since the start of incubation of lymphocytes in the serum. Constant changes in shape of the cells are obvious in these photographs taken 15 sec. apart. The rate of movement of lymphocytes is a delicate index of "vitality." Taken from a film sequence, phase-contrast.

of the cytoplasm. The cell volume often increases to twice its normal size (FIGURE 3), with a rapid clearing (liquefaction) of the cytoplasm. Nuclear pyknosis becomes very marked in the final stages. Actual lysis of the cells thereafter has not been observed.

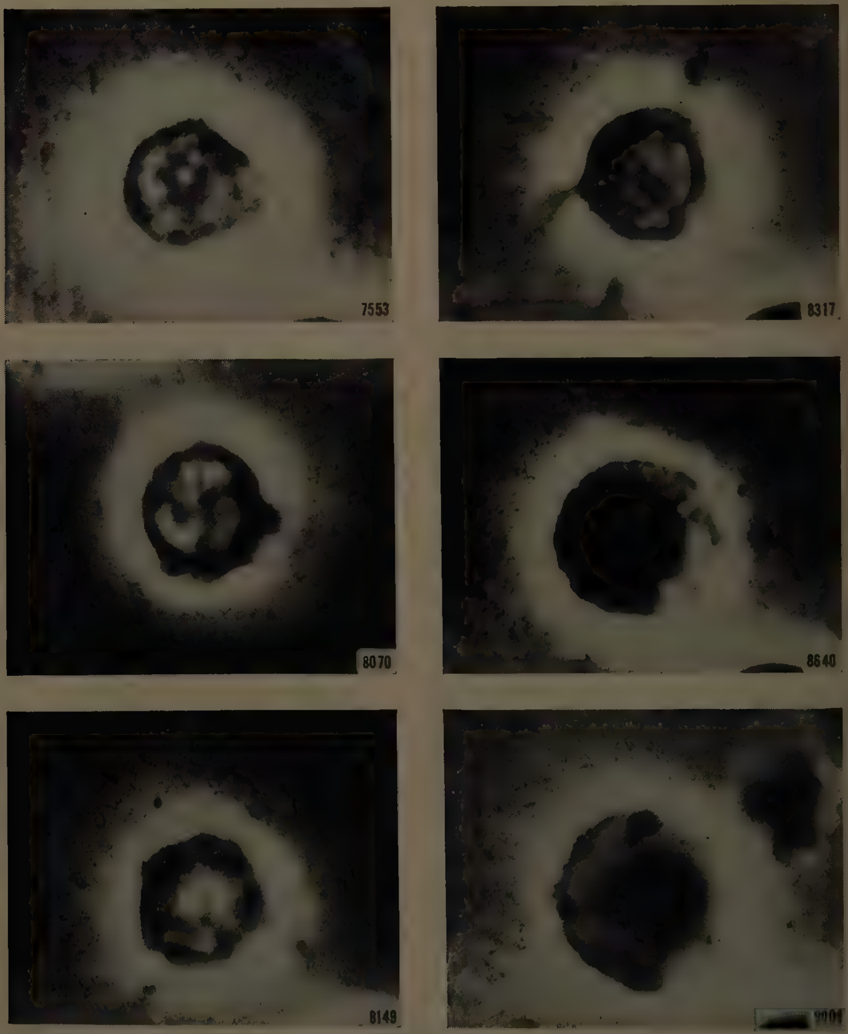

FIGURE 2.

FIGURES 2 and 3. The sequence of events in the death of a lymphocyte produced by serum from a chicken injected with pooled homologous splenic tissue. The numbers indicate the time in seconds that the lymphocytes were incubated in the serum. It may be seen that the cells are rounded as compared to FIGURE 1. By the start of these sequences, the cells have become stationary, but they were all still making small pseudopodial movements. Although the nucleus is not normally well demarcated from the cytoplasm, it becomes gradually prominent and optically dark. Pyknosis of the nucleus is evident in FIGURES 2 and 3. The swelling of the cytoplasm can also be seen from the sequence. FIGURE 3 shows a cell which developed filaments just before death. The cell continued to move, even though rounded until 8317 seconds after incubation. Swelling progressed after the cessation of cell movement.

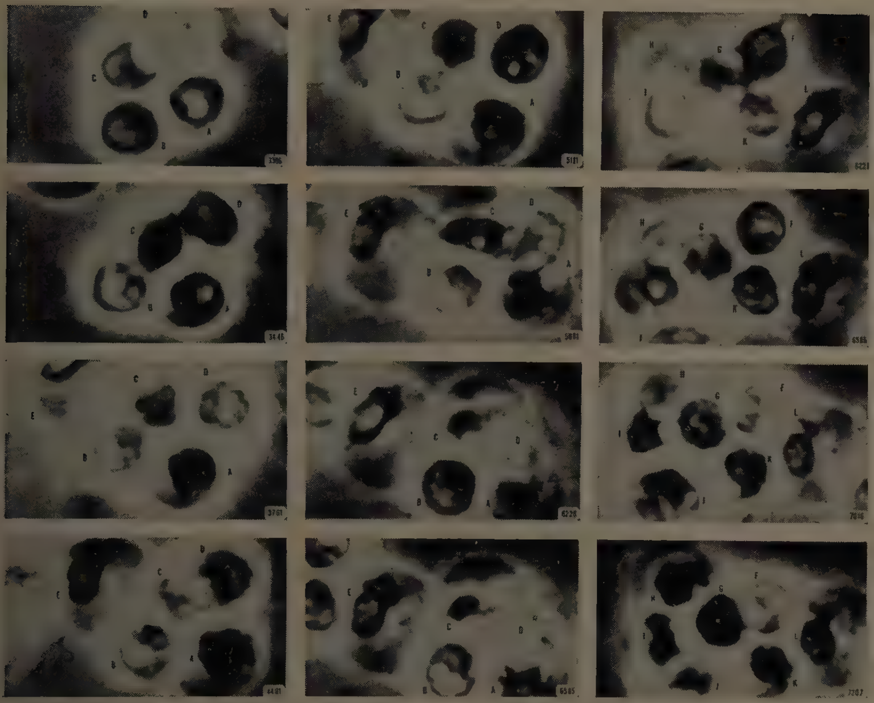

FIGURE 3.

Discussion

Humoral cytotoxic antibodies have been shown to be effective *in vitro* against various homologous tumor cells (see reviews by Snell[14] and Gorer[6]). Although such antisera are also effective against normal lymphoid cells [7,8,9] sera from animals injected with normal homologous cells have generally not been cytotoxic *in vitro*.[1,11] Immunization by skin grafting[11] or injection of skin extracts together with skin grafting[1] did not yield detectable humoral antibodies against tissue-cultured skin cells. In the present experiments, sera from animals injected with pooled homologous splenic cells have been shown to have a definite toxic effect upon homologous lymphocytes. It is evident from TABLE 1 that sera from immunized animals cause a marked decrease in viability of lymphocytes in most instances. Only when the time of incubation was short or when the time between the last immunization and bleeding was long, did there seem to be no decrease in the percentage of viable cells.

Since we have experienced some difficulty in the use of stains for determination of viability of lymphocytes,[7] time-lapse cinematographs were taken of the cells, facilitating the use of motility as the index of viability. De Bruyn[4] has shown by time-lapse studies that lymphocytes undergo an active locomotory phase (polarized phase) and a "rest period" (depolarized phase). He also has pointed out that during the so-called rest period the cells are not entirely stationary, but are constantly moving irregularly for short distances, and many small pseudopodia are seen to arise continuously. Thus, observation with time-lapse cinematography for as short a period as 15 min. is sufficient to detect the normal movements indicative of the viability of a lymphocyte. Our films clearly show that, once this movement ceases, irreversible processes associated with post-mortem changes occur. It is possible, therefore, to count with considerable accuracy the exact number of viable cells present for each field photographed.

The cytological changes that occur in the lymphocytes are quite similar to those noted by Schrek and Preston[13] in the Bagg lymphosarcoma cells incubated with sera from rats with regressed tumors. Initially, upon contact with immune serum, the cells rounded up, the nucleus became gradually visible and contained a clear area, and coarse granules attached to the nuclear wall. The nuclear membrane then collapsed, liberating the contents into the cytolyzed cell. Bessis[3] has described somewhat similar changes occurring in the lysis of white blood cells produced by immune sera from patients with various diseases, as have Kalfayan and Kidd[10] with respect to Brown-Pearce tumor cells. Cytotoxins to leukocytes found in humans under various conditions have been reviewed by Walford.[18]

The sera from animals injected with splenic cells also produce marked agglutination of lymphocytes, as described previously.[16,17] Although no direct comparisons have been made, agglutination can be detected readily at high dilutions of serum, while cytotoxicity has been seen only at low dilutions of serum. From the present experiments, little can be said about the role of these *in vitro* cytotoxins in the normal rejection of homografts, except that some evidence is provided against the widely held belief that *in vitro* cytotoxins cannot be shown following normal tissue homografts.

Summary

Sera from animals injected with pooled homologous splenic cells have been shown to cause cytotoxic effects upon normal lymphocytes *in vitro*. Viability of the cells was assessed by the determination of the percentage of motile cells as shown by time-lapse cinematography of each culture. The percentage of motile cells in immune serum has been decreased by as much as 100 per cent from the percentage of motile cells in normal serum. The degenerative changes in lymphocytes caused by immune sera are also described.

Acknowledgments

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References

1. Allgöwer, M., T. G. Blocker, Jr. & B. W. D. Engley. 1952. Some immunological aspects of auto- and homografts in rabbits, tested by *in vivo* and *in vitro* techniques. Plastic and Reconstruc. Surg. **9:** 1–21.
2. Bessis, M. 1956. *In* Cytology of the Blood and Blood-Forming Organs. : 266–271. Grune & Stratton. New York, N. Y.

3. Bessis, M. 1954. Phagocytosis and other phenomena of sensitized red cells, white cells and platelets. Vox Sang. **4:** 177–180.
4. De Bruyn, P. 1945. The motion of the migrating cells in tissue cultures of lymph nodes. Anat. Record. **93:** 295–315.
5. Feldman, M. & L. Sachs. 1957. The antibody response to successful tumor homografts. J. Natl. Cancer Inst. **18:** 529–553.
6. Gorer, P. A. 1956. Some recent work on tumor immunity. Advances in Cancer Research. **4:** 149–186.
7. Gorer, P. A. & P. O'Gorman. 1956. The cytotoxic activity of isoantibodies in mice. Transplantation Bull. **3:** 142–143.
8. Gorer, P. A. & E. A. Boyse. 1959. Some reactions observed with transplanted reticulo-endothelial cells in mice. Congr. et Coll. Univ. Liege. **12:** 193–206.
9. Hellström, K. E. 1959. Cytotoxic effect of isoantibodies on mouse tumor cells *in vitro*. Transplantation Bull. **6:** 411–416.
10. Kalfayan, B. & J. G. Kidd. 1953. Structural changes produced in Brown-Pearce carcinoma cells by means of a specific antibody and complement. J. Exptl. Med. **97:** 145–162.
11. Medawar, P. B. 1948. Tests by tissue culture methods on the nature of immunity to transplanted skin. Quart. J. Microscop. Sci. **89:** 239–252.
12. Rose, G. 1954. A separable and multipurpose tissue culture chamber. Texas Rept. Biol. and Med. **12:** 1074–1083.
13. Schrek, R. & F. W. Preston. 1956. Toxicity of homologous immune serum to transplantable tumor. Studies using phase microscopy and cinematography. J. Natl. Cancer Inst. **16:** 1021–1045.
14. Snell, G. D. 1957. Incompatibility reactions to tumor homotransplants with particular reference to the role of the tumor. Cancer Research. **17:** 2–10.
15. Terasaki, P. I. 1959. Identification of the type of blood cell responsible for the graft versus host homograft reaction in chicks. J. Embryol. Exptl. Morphol. **7:** 394–408.
16. Terasaki, P. I., J. A. Cannon & W. P. Longmire, Jr. 1959. Antibody response to homografts. I. The technique of lymphoagglutination and the detection of lymphoagglutinins upon injection of homologous splenic tissue. Proc. Soc. Exptl. Biol. Med. **102:** 280–285.
17. Terasaki, P. I. 1959. Antibody response to homografts. II. Preliminary studies of the time of appearance of lymphoagglutinins upon homografting. Am. Surg. **11:** 896–899.
18. Walford, R. L. 1960. Leucocyte Antigens and Antibodies. : 81–83. Grune & Stratton. New York, N. Y.

A DEMONSTRATION OF A CYTOTOXIC EFFECT *IN VITRO* FOLLOWING THE REJECTION OF SKIN GRAFTS BY THE RABBIT*

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The *in vitro* demonstration of "circulating antibodies" to donor tissue following the rejection of normal tissue homografts has been a matter of controversy for some time. Conflicting reports have appeared regarding the demonstration of both circulating serum antibodies and fixed cellular antibodies. Following the rejection of skin grafts by mice, hemagglutinins for donor erythrocytes in dextran-recipients' serum mixtures have been described by Amos *et al.*[1] The relation of such agglutinins to the rejection of transplanted tissue, however, has been put in question by the results of Hildemann and Medawar.[2] The latter found that crude splenic nuclear fractions can incite skin transplantation immunity to mice without producing a detectable titer of hemagglutinins. Such fragments also have little if any power to absorb antibodies from sera in which they have been caused to appear by other means. It has been suggested that the relation of the development of hemagglutinins to the transplantation immunity is due to the sharing of the H_2 antigen by the red cells and tissue cells of the mouse.[3] This sharing of these antigens does not occur in the rabbit.[4] Simonsen *et al.*,[5] however, found agglutinins to donor red cells in the sera of dogs that had rejected kidney homografts. "Antibodies" have been found in the sera of rabbits by Bollag,[6] who described increased turbidity when the sera of rabbits that had rejected skin grafts were incubated with an extract of donor tissue. Bollag's results have not been confirmed by other observers.

The work of Weaver *et al.* suggested that the factor responsible for the death of transplanted cells was to be found in the sensitized lymphocytes of the recipient.[7] The publications of Voisin and Maurer[8] and Berrian and Brent[9] tended to confirm this. The assay in the latter two instances, however, was the accelerated rejection of skin grafts, an *in vivo* technique. *In vitro*, the demonstration of the role of cell-bound antibodies has been less consistent. Donor cells grown in tissue culture showed no abnormalities when incubated with recipient sera with or without cells from the regional lymph node cells of recipient animals that had rejected normal tissue grafts.[10-13]

In our earlier investigations we too were unable to demonstrate homograft immunity consistently by *in vitro* techniques. We investigated this immunity by means of the precipitin technique, utilizing the sera of rabbits that had rejected one or more skin homografts. As antigen we used a sonic lysate of donor leukocytes prepared after the manner of Billingham.[11] Such a lysate had been shown to have antigenic activity *in vivo* and thus might be a suit-

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able component of a precipitin reaction with the serum from immunized recipients. With this technique, however, our results were inconsistent. Similarly, we were unable to demonstrate immunity utilizing the technique of Stavitsky.[14] In these tests tanned red cells were sensitized with the lysate of donor cells and incubated with the serum of postgraft recipient animals.

We next attempted to demonstrate a cytotoxic effect of recipient serum and cells upon whole donor leukocytes, using the technique of Gorer and O'Gorman.[15] These authors demonstrated the cytotoxic activity of iso-antibodies from mice immunized by tumor cells from a homologous strain, when recipients' sera were incubated with normal donor splenic cells. Utilizing first a heterologous system, we found that the sera of rabbits immunized by suspensions of human white cells contained precipitins for the lysate of human leukocytes. In addition, the sera were strongly cytotoxic for whole human leukocytes, as evidenced by the uptake of trypan blue by the donor cells. In this system 90 per cent of the donor cells were stained; also, they showed the swelling and fragmentation commented upon by Gorer and O'Gorman.[15] However, sera drawn from recipient rabbits 4 to 10 days after the rejection of the second homograft showed neither precipitins for lysates of donor cells nor a consistent cytotoxic effect upon whole donor leukocytes either from peripheral blood or from peritoneal washings. The results, although not statistically significant, showed a less consistent cytotoxic effect when immune recipient sera plus recipient lymphocytes were incubated with donor cells than when recipient and donor cells alone were mixed, suggesting a possible inhibitory effect of the immune serum.

Under the circumstances of our experiment, however, the recipient cells utilized were either those of the general white cell population (in the case of whole blood) or the general lymphocyte population (in the case of peritoneal washings). Berrian and Brent[9] and Scothorne[16] have suggested that it is largely the lymphocyte from the regional lymph node that is responsible for the rejection of a homograft. However, recent work by Perry *et al.*[17] and Gowans[18] suggests that there is a constant turnover of lymphocytes, and that the lymphocytes in the blood recirculate into and from the thoracic lymph via the lymph nodes. Thus, if homograft rejection is mediated only by the sensitized lymphocytes and those predominately from regional nodes, the proportion of lymphocytes in the general circulation sensitized specifically for the antigen of the skin donor must be small. Thus, it might have been the small number of these cells in our previous test systems that accounted for the negative results. To overcome this difficulty it occurred to us that, if the sensitized lymphocyte is attracted by the specific antigen, it might thus be "trapped" in larger numbers by utilization of specific donor antigen. With this in mind, the following experimental design was employed (FIGURE 1).

Methods

Large skin homografts (5 by 7 cm.) were exchanged between 7- to 9-lb. rabbits of several breeds from different litters. Following rejection of the second homograft, dissociated epidermal cells were prepared from the skin of one of the animals (designated as donor D for this experiment) by trypsinization according to the method of Billingham.[11] D cells were injected in equal amounts intraperitoneally into both donor (D) and recipient (R) animals. Twelve to 18 hours later 100 to 200 ml. of a 1 per cent solution of glycogen in 0.9 per cent saline was injected intraperitoneally into both animals. One to 2 hours later this fluid was removed by inserting into the peritoneal cavity a small multiholed plastic catheter. The fluid so obtained was rich in white cells, approximately 90 per cent of which were lymphocytes. Only rarely were red cells found. The cells were suspended in normal rabbit sera or pregrafting recipient sera diluted 1:5 with buffered Ringer's solution to give a total leukocyte count of 20,000 to 50,000 cells per milliliter.[3] One-tenth ml. of "donor" cell suspension plus 0.1 ml. of a 1:5 dilution of preimmune "recipient" serum and 0.1 ml. of a 1 to 2 dilution of desiccated pooled guinea pig serum were mixed (tube D) and 0.1 ml. of recipient cells similarly treated (tube R). In a third tube 0.05 ml. each of recipient cells

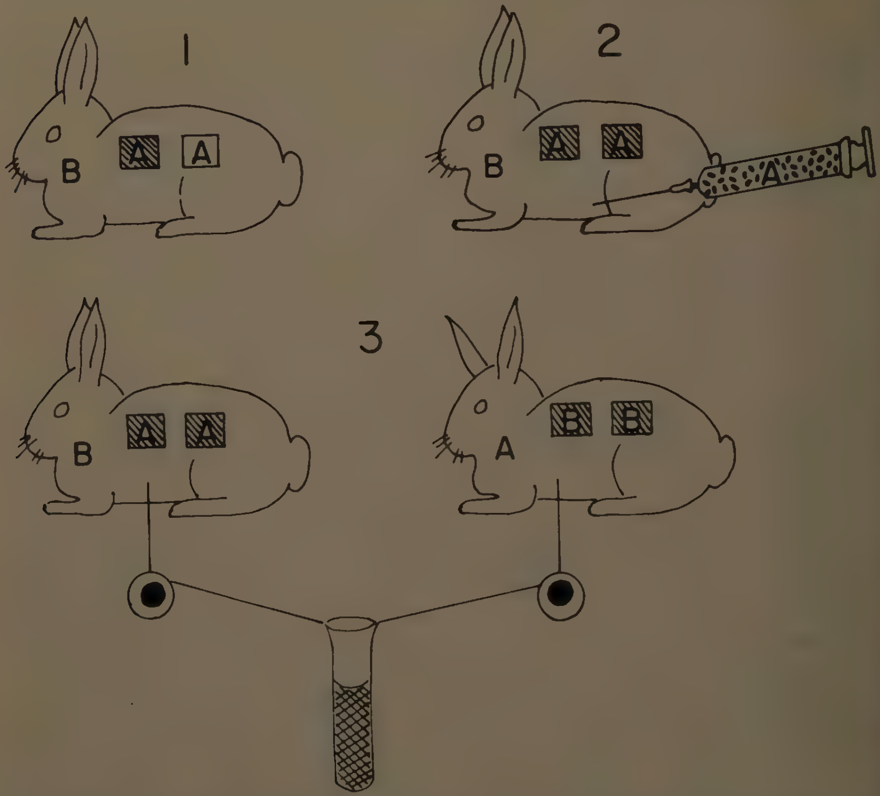

FIGURE 1. (*1*) Recipient animal (*B*) has received two skin homografts from donor animal (*A*). (*2*) Following rejection of the skin graft (*B*) is challenged with trypsinized epithelial cells from (*A*). (*3*) Cells are harvested 12 to 18 hours later by peritoneal irrigation from both animals and antibody separately and together as described in methods.

and donor cells was added to recipient serum and complement (tubes D and R). The tubes were incubated for one hour at 37° C.; then part of the mixture from each tube was diluted 1:5 in a 1000:2000 dilution of eosin or trypan blue in Tyrode's solution. A drop of the resulting mixture was placed in a counting chamber and counted for stained cells. Two hundred or more cells were counted by at least 2 observers, one of whom was unaware of the origin of the mixture counted. The percentage of stained cells counted by each observer agreed within 5 per cent when the mixtures were counted at the same time. As controls, 2 rabbits with 6- to 10-day autografts were injected intraperitoneally with their own trypsinized epithelial cells. After 18 to 24 hours these cells were harvested by the method described. In 4 experiments normal (ungrafted) rabbits were injected with trypsinized skin cells from other animals and with their own skin cells. Lymphocytes from the peritoneal cavity of both groups of animals were incubated separately and together. When the mixtures were counted at 1 and 4 hours after the termination of incubation, the percentage of stained cells in each tube had increased, but the ratios remained the same between tubes.

Results

In none of the control animals did more than 12 per cent of the cells manifest a cytotoxic effect, as evidenced by the uptake of trypan blue. Fifteen experiments were performed in which 2 skin grafts were crossed between 2 rabbits and the trypsinized epithelial cells from 1 injected intraperitoneally into both animals. The results of these experiments are shown in TABLE 1. The application of a *t* test to these values shows that the percentage of recipient cells stained after exposure to trypsinized epithelial cells alone was significantly greater in the homologous than in the autologous system.

In two preliminary experiments intraperitoneal challenge of both donor and recipient by sonic lysate of donor cells resulted in significant staining of recipient but not donor cells. After incubation of the recipient cells with donor cells this effect was even more marked. Since exactly equal quantities of donor and recipient cells were mixed together in only two of the fifteen experiments, the D plus R combination is apparently, but not statistically, significantly greater than R alone. This might be accounted for by the dilution of stained R cells by unstained D cells. If one assumes an equal proportion of D and R cells with the average staining, the combination D plus R is greater than R alone at the 95 per cent confidence level. It seems apparent, therefore, that although some damage occurred to recipient cells during contact, *in vivo* incubation with donor cells produced further damage in the mixture. Under the conditions of our experiment it cannot be stated whether this further damage occurred to recipient or to donor cells, or to both.

Discussion

The ability of living cells to exclude trypan blue and eosin has been shown to be a sensitive and quantitatively accurate test of their viability.[19] Conversely, the uptake of these dyes indicates cell damage, and this occurs at an earlier stage than gross morphologic change. Experienced observers counting the same field agree upon the percentage of stained cells within an extremely narrow range (plus or minus 5 per cent). These considerations make the cytotoxic tests described above a simpler, more rapid, and probably more accurate test than the morphology of cells in tissue culture. The results presented above suggest that sensitized lymphocytes from rabbits immunized by the rejection of two skin grafts participate in a reaction *in vitro* with donor cells that results in damage to both cells. The damage would seem to be as much (or more) to the recipient cells as to the donor cells. This interesting fact is not generally discussed, but it is commented upon by Weaver and his collaborators,[7] Billingham,[11] and Medawar.[10] When no donor antigen was injected intraperitoneally prior to harvesting recipient cells, no consistent cytotoxic effect was observed. The positive results following the intraperitoneal injection of donor cells suggest that donor antigen is necessary to "attract" an adequate number of sensitized cells from the general round cell population. Since recipient cells alone showed more staining than donor cells following injection of donor epithelial cells into both animals, it seems possible that the cytotoxic effect begins *in vivo*. The mixture of R and D cells showed a higher number of cells stained than did R cells alone when incubated. These results, however, are not statistically significant for the number of experiments done. However, in twelve of the experiments the numbers of cells in the donor and recipient suspensions were not quantitatively equal to each other. For the 15 experiments, therefore, the smaller percentage of donor cells stained must have weighted the results in the direction of less difference between R cells alone and the mixture R plus D. If, however, we assume for each 0.05 ml. of cells that the number of cells stained is represented by the average actually determined (TABLE 1), the value for D plus R becomes significantly higher ($P < 0.05$) than that calculated for a simple mixture of D plus R in equal proportions. Since we are unable to differentiate donor cells from recipient cells in the counting-chamber mixture, these results do not tell us whether the addition of donor cells results in a cytotoxic effect on the added donor cells or simply an increased cytotoxic effect to the recipient lymphocytes. It might be argued that the demonstration following incubation of the cells is not truly an *in*

TABLE 1

PERCENTAGE OF STAINED CELLS

	D	R	D + R
Average	10.8	19.1	32.3
Standard error of mean	2.3	3.1	6.1
No. of experiments	15	15	15

1) $R > D$ $(P < 0.05)$
2) $(D + R) > D$ $(P < 0.01)$
3) $(D \pm R) > R$ $(P < 0.05)$

D = Donor, R = Recipient.

vitro test but simply the accentuation of damage to recipient cells by incubation at 37° C. for 1 hour. This is suggested by the fact that recipient cells incubated alone after harvesting show a significantly greater staining than do donor cells. It is suggested also by the fact that prolonging the incubation of any cell type causes an increased uptake of stain as the cells die *in vitro*. However, the percentage of cells stained in the mixture appears to be greater than can be accounted for by dilution by nonstained donor cells alone. This fact must be substantiated by further experiments.

That the injury to the recipient cell is of prime importance is indicated by our preliminary experiments, which show that a nonviable lysate injected intraperitoneally will also cause a higher percentage of staining of recipient than donor cell. This is consistent with the observation of Berrian and Brent[9] that sensitized cells and lysate of donor cells combine in such fashion as to inactivate the former. Berrian, however, observed no morphologic change in the recipient cells, although he did not employ the cytotoxic technique.

Billingham[11] has suggested that the mechanism of homograft destruction may be via some product of an interaction between donor and recipient cells at the site of the graft. Such interaction involving the destruction of both cells seems possible from the results presented here. Converse and Rapaport,[20] employing stereomicroscopy, have shown that an early stage in the rejection of a homograft is a retardation of blood flow and the agglutination of cells in the graft capillaries. Amos[21] has reported leukoagglutination as a result of homograft immunity. In some of our preparations showing the greatest cytotoxic effect there also is evidence of leukocyte agglutination. It is possible that leukocyte agglutination (along with erythrocyte agglutination) may be responsible for the thromboses and ischemia that occur in skin grafts. However, it is also possible that the leukoagglutination is a result of nonspecific changes and damage to cells and graft, or both. This phenomenon has been suggested by Simonsen,[5] who noted panagglutination of erythrocytes following kidney homograft rejection. It is well known that direct damage to vascular endothelium may cause agglutination and adherence of cells within the capillaries.

Summary

When cells harvested by peritoneal washing from rabbits who have rejected two skin grafts are incubated with donor cells similarly obtained, a cytotoxic effect is evidenced in the cells of the mixture.

The cytotoxic effect is manifest only if the recipient has been challenged twelve to eighteen hours previously by the intraperitoneal injection of trypsinized donor epithelial cells or sonic lysate of donor cells.

Recipient cells alone following this challenge show a significantly greater cytotoxic effect than do donor cells alone, but less effect than the mixture.

The percentage of stained cells in donor cells alone is within the normal range for this technique, as are cells similarly prepared from autografted and nongrafted rabbits.

It is concluded that homograft rejection in the rabbit may be demonstrated by a cytotoxic effect elicited *in vitro* by the incubation of donor and recipi-

ent cells. Damage to the recipient cells is a major factor in this effect and begins *in vivo* following the intraperitoneal injection of donor cells.

References

1. Amos, D. B., P. A. Gorer, B. M. Mikulska, R. E. Billingham & E. M. Sparrow. 1954. An antibody response to skin homografts in mice. Brit. J. Exptl. Pathol. **35:** 203.
2. Hildemann, W. H. & P. B. Medawar. 1959. Relationship between skin transplantation immunity and the formation of humoral isoantibodies in mice. Immunology **2:** 44.
3. Medawar, P. B. 1959. Iso-antigens. Biological problems of grafting. A symposium. Univ. Liége. Liége, Belgium.
4. Medawar, P. B. 1956. Immunity to homologous grafted skin. II. The relationship between the antigens of blood and skin. Brit. J. Exptl. Pathol. **27:** 15.
5. Simonsen, M., J. Buemann, A. Gammeltoft, F. Jensen & K. Jorgensen. 1953. Biological incompatibility in kidney transplantation in dogs. I. Experimental and morphological investigations. Acta Pathol. Microbiol. Scand. **32:** 1.
6. Bollag, W. S. 1956. Demonstration of antibodies following homografts. Transplantation Bull. **3:** 43.
7. Weaver, J. M., G. H. Algire & R. T. Prehn. 1955. The growth of cells *in vivo* in diffusion chambers. II. The role of cells in the destruction of homografts in mice. J. Natl. Cancer Inst. **15:** 1737.
8. Voisin, G. A. & P. Maurer. 1957. Studies on the role of antibodies in the failure of homografts. Ann. N. Y. Acad, Sci. **64**(5): 1053.
9. Berrian, J. H. & L. Brent. 1958. Cell bound antibodies in transplantation immunity. Ann. N. Y. Acad. Sci. **73**(3): 654.
10. Medawar, P. B. 1948. Tests by tissue culture methods on the nature of immunity to transplanted skin. Quart. J. Microscop. Sci. **89:** 239.
11. Billingham, R. E. & E. M. Sparrow. 1954. Studies on the nature of immunity to homologous grafted skin with special reference to the use of pure epidermal grafts. J. Exptl. Biol. **31:** 16.
12. Allgower, M., T. G. Blocker, Jr. & B. W. Engley. 1952. Some immunological aspects of auto- and homografts in rabbits, tested by *in vivo* and *in vitro* techniques. Plastic and Reconstruction Surg. **9:** 1.
13. Miller, T. G. 1956. *In vitro* studies on the iso-antigen system in mice. J. Natl. Cancer Inst. **16:** 1473.
14. Stavitsky, A. B. 1954. Micromethods for the study of proteins and antibodies. I. Procedure and general applications of hemagglutination-inhibition reactions with tannic acid and protein-treated red blood cells. J. Immunol. **72:** 360.
15. Gorer, P. A. & P. O'Gorman. 1956. The cytotoxic activity of isoantibodies in mice. Transplantation Bull. **3:** 142.
16. Scothorne, R. J. 1957. Studies on the response of the regional lymph nodes to skin homografts. Ann. N. Y. Acad. Sci. **64**(5): 1028.
17. Perry, S., C. G. Craddock, G. Paul & J. S. Lawrence. 1959. Lymphocyte production and turnover. A.M.A. Arch. Intern. Med. **103:** 224.
18. Gowans, J. L. 1959. The recirculation of lymphocytes from blood to lymph in the rat. J. Physiol. **146:** 54.
19. Hanks, J. H. & J. M. Wallace. 1958. Determination of cell viability. Proc. Soc. Exptl. Biol. Med. **98:** 188.
20. Converse, J. M. & F. T. Rapaport. 1956. The vascularization of skin autografts and homografts. An experimental study in man. Ann. Surg. **143:** 306.
21. Amos, B. B. 1953. The agglutination of mouse leucocytes by iso-immune sera. Brit. J. Exptl. Pathol. **34:** 464.

POSSIBLE RELATIONSHIPS BETWEEN THE CYTOTOXIC EFFECTS OF ISOANTIBODY AND HOST CELL FUNCTION*

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Antibodies usually can be detected in the serum of mice that have recently rejected a homograft. This reversal of the findings of an earlier generation is now well substantiated and has been accomplished chiefly through persistent efforts to improve the methods available for antibody detection.[1-6]

For many years there was doubt as to the existence of such isoantibodies; interest has now shifted to discussion of the significance of the antibodies formed, their functional relationship to the host cells found in proximity to the graft, and to the differences between humoral antibody and hypothetical cell-bound antibodies.

Resolution of many of the problems concerning the relationship between host cells and antibody during graft rejection is difficult because lymph node and spleen cells (the usual sources of immune cells in transplantation experiments) liberate circulating antibody; there is therefore some uncertainty as to what each component is doing. Transfer of large quantities of regional lymph nodes to a nonimmune animal confers a state of immunity almost as complex as that existing in the originally immunized host.[7] Winn's modification of the technique whereby immune cells are mixed directly with the tumor suspension before injection has already given further quantitative information.[8] It is probable that further experiments of this nature will resolve a number of questions raised by the earlier experiments of Mitchison[7] (*see also* Gorer[9]).

While recognizing that the reactions occurring during graft rejection involve a variable number of components and that these components must interact, sometimes synergically and sometimes antagonistically, it seems logical to simplify the system as far as is practicable. Humoral antibody can be separated readily from cells, whereas it is difficult to obtain cells free from antibody. Antibody activity therefore has been defined with greater precision.

This paper surveys the increasing evidence for antibody participation in homograft rejection, discusses some observed anomalies of both antibody and immune cell activity, and presents a hypothesis to explain why antibody, complement, immune cells, enzymes, and enzyme inhibitors may all be interdependent.

Humoral factors. Antibody activity was clearly demonstrated against homografts in rats by Lumsden[10,11] (*see also* the review by Moppett[12]). Criticism by Ludford[13] and by Phelps[14] largely obscured the significance of much of Lumsden's excellent work, and the overenthusiasm of his conclusions helped to push his work into obscurity. Gorer painstakingly re-established the validity of many of Lumsden's ideas and found, first in rabbits and then in

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mice of different strains, antibodies that, when mixed with leukemic cells, would prevent the appearance of a tumor and could be readily demonstrated by hemagglutination.[1,2]

Later even more convincing evidence for antibody activity was obtained when immunity to transplanted leukemias such as E.L.4 could be transferred to a nonimmune host with small volumes of antibody[3] even if the antibody was given as long as seven days before the tumor was injected. With the development of *in vitro* cytotoxicity tests, dependence upon hemagglutinin titration has been greatly reduced.

The hemagglutinating properties of isoantibody, however, have served two useful purposes:

First, they have been invaluable in studying the genetic configuration of the animals. Knowledge of the complexity of the H-2 locus, in particular, never could have been gained in so detailed and precise a manner by conventional genetic methods. Although this type of study is by no means completed and much useful genetic information is still to be gained, it has shown that individual antigens are frequently shared between strains and that immunity against one foreign strain is consequently likely to be reflected in many other strains.[15-17]

Second, hemagglutination provides a useful although not absolute indication of the potency of a serum, since a high-titer hemagglutinin is likely to be associated with a high titer of cytotoxic activity. Fluctuations in level are easily followed, and small amounts of antibody can be detected more readily as hemagglutinin than as cytotoxin.[6]

The ease with which passive immunity can be transferred and cytotoxicity demonstrated *in vitro* has assured most investigators that, under the appropriate conditions, humoral antibody is intensely lytic for certain types of cell; it is not evidence that antibody acts without augmentation of its effects by host cells in the nonimmune mouse, or even that it can do so. Relatively little is known about natural immunity to homografts. More attention was devoted to this aspect of the problem when only random-bred mice were available; there seems to be less interest in natural immunity in recent times. Moore and Oliver found strong evidence of large mononuclear proliferation and tumor suppression reversible by cortisone in the lung and liver of normal Swiss mice injected intravenously with Ehrlich ascites cells (Moore and Oliver, to be published).

Further evidence for the *in vitro* activity of antibody without host cells was therefore sought, first by growing cells in diffusion chambers designed to exclude the vast majority of host cells, and then by seeing whether graft destruction without host-cell proliferation occurred in the peritoneal cavity of passively immunized mice (this latter study has not yet been completed, since such interesting results, described below, were found in nonimmune hosts).

Growth curves for a number of ascites tumors sealed inside diffusion chambers and placed in the peritoneal cavity of both homologous and isologous strains were first established. Growth rates were essentially the same in both, showing that there was no evidence of inhibition by natural humoral immune factors.[18] Some experiments were performed later with initial populations of less than 5000 DBA/2 thymoma cells per chamber (Amos and Dietter, unpublished data). This appeared to be the lowest limit for survival inside the chamber under the conditions of the experiment. Even with such a dose which is one twentieth that required to produce a tumor in a homologous mouse, there was no difference in growth rate between foreign and susceptible lines. In the absence of an obvious leak, host cells in any appreciable number do not enter adequately sealed diffusion chambers of the type used.

From these experiments it was concluded that any natural humoral immunity was unlikely to influence the effect of antibody on transplanted lymphoma cells growing in diffusion chambers.

Antibody passes rapidly from the peritoneal cavity into the blood, but more slowly in the reverse direction. At equilibrium the concentration in the peritoneum tends to be about one half that of the serum[18] (*see also* Algire[19] and Algire *et al.*[20]). Cells from different ascites tumors were grown for several days in chambers, and the appropriate isoantibody was injected into the peritoneal cavity. Antibody could be demonstrated inside the chamber within 1 hour, and a profound fall in cell numbers had occurred within 4 hours. The response was roughly proportional to a function of the amount of antibody injected, but tended to be transient, the surviving cells growing at the rate of untreated control cells within 48 hours. Depression of growth rate could be maintained by daily injections of antibody but, even after 5 days' suppression, normal growth rates were resumed upon stopping antibody injections. Antibody levels in the chamber as well as in the serum and ascites fluid were high even though the cells were increasing rapidly, and it was thought at first that immunoselection of a resistant population might have occurred. The surviving cells, however, still were susceptible to the cytotoxic effect of other isoantibodies in *in vitro* tests using normal guinea pig serum, and the supernatant from the chambers was also cytotoxic for normal DBA/2 thymoma cells and for the surviving chamber population.[21]

It appeared that the limiting effect was due to lack of complement, and a number of experiments were designed to increase the efficiency of antibody by adding normal serum.[22] Rather surprisingly, 1 ml. of mouse serum was as effective as 1 ml. of guinea pig complement *in vivo*, although it has long been known that guinea pig serum is very much more active *in vitro* (*see* Levy[23]). The difference between the *in vivo* and *in vitro* results is explicable by the anticomplementary effect of mouse serum for guinea pig complement. Large amounts of zymosan abolished the effect of antibody, while the levels of complement in chambers in untreated animals were found to be only about one tenth of that in the serum. The chambers themselves were not markedly anticomplementary *in vitro*.

The limitation of this type of test system appears to be in the level of complement attained inside the chamber. Injections of large volumes of normal serum are impracticable, since the abdominal wound made in introducing the chamber tends to reopen. Lyophilized serum reconstituted in less than the original volume is hyperosmotic and dehydrates the cells in the chamber, while our attempts to concentrate mouse complement have not yet been successful.

Although, for the reasons stated, the diffusion chamber experiments have not been completed since the ultimate cytotoxic potential of antibody in the presence of complement excess has not been reached, there is sufficient evidence to show that host cells are not necessary for lysis of lymphoma cells, that the immediate activity of antibody in diffusion chambers compares well with that found in passive immunity experiments, and that heterologous complement is not required *in vivo.*

While antibody is cytolytic for lymphoma cells and for spleen and thymus cells and for many, but not all, marrow cells, other tissues show varying degrees of resistance to these same antibodies.[24,25] In our laboratory an isoantibody against C3H ascites sarcoma MClM will cause relatively little damage to MClM *in vitro*, while it will cause complete destruction of 6C3HED. Mouse isoantibodies are not complemented by mouse serum *in vitro* and, as undiluted mouse serum is anticomplementary to the guinea pig, the ultimate limits of antibody activity have not been reached *in vitro* any more than they have *in vivo.* Nevertheless, the failure of isoantibody to cause any apparent damage to such tumors as Hauschka's Ehrlich clone E2, while under the same conditions 95 to 100 per cent of lymphoma cells are completely destroyed, illustrates the fundamental difference in sensitivity.

We thus have the paradox of an antibody that is regularly formed at the time of homograft rejection, that has strong cytotoxic activity for lymphoid and certain other cells, but that will often not damage the cells against which it is induced.

Cellular factors. An examination of some of the facts known about the cellular response also reveals a rather confusing situation. Loeb laid considerable emphasis upon the complexity of the infiltration following the transplantation of compound tissues.[26] Different organs elicit different responses. Rejection is not invariably accompanied by infiltration or even by an accumulation of cells in the graft area.[26,27] The predominant reactive cell type varies with different types of tissue transplanted and with different host-donor relationships.[24,28,29]

The differences between the host-cell types invading a graft have provided a perplexing and rarely stressed situation. Either these different types of cells have something in common, or else there are as many different mechanisms in graft rejection as there are cell types.

Most authors since the time of Murphy[29] favor the lymphocyte as the active cell, and there is strong support for this view. There is a rise in circulating lymphocytes at about the time of homograft rejection (in the mouse many of these are actually monocytoid), often an accumulation of cells, including and sometimes predominantly lymphocytes in the graft area, and lymph nodes have been shown to be a seat of antibody production. There is still no information about the mode of action of these cells. Experiments involving the adoptive transfer of immunity have done little to clarify the complexity since the essentials for the reactions, plasma cells, lymphocytes, histiocytes, and the stimulus and ability to manufacture antibody are all transferred.

Many authors believe that lymphocytes are carriers of bound antibody (*see* Brent *et al.*[30]); although this is a venerable idea, the nature of this bound reactant has never been demonstrated. Attempts at extraction have failed to reveal any great concentration of isoantibody; on a wet-weight basis there is more isoantibody expressed as hemagglutinin in lung or in the homogenized eviscerated carcass than in the lymph nodes and spleen of a hyperimmune mouse (Amos, unpublished data). Similarly, it is difficult to see why an excess of humoral antibody should be ineffective while the same antibody bound to a lymphocyte should be so very active. Medawar has proposed recently that the "anti substances" in lymphocytes may differ profoundly from serum antibodies, but he offers no suggestion as to what they might be.[31] While there is no clear evidence of the function of immune cells, there is no doubt that they are essential for the usual type of immune response.

The experiments of Weaver, *et al.*[32] are perhaps the strongest evidence for the direct participation of lymphocytes: "Whenever target cells were found together with lymphocytes from mice immunized to the target cells, both types of cells were degenerating or dead." These authors found no evidence of phagocytic activity and no lysis if the lymphocyte and tumor were separated, until after the destructive reaction had started, when "tumor cells and lymphocytes, away from the zone of contact between these two types of cells, were also found degenerating"[32] (*see also* Weaver[33]). Similar experiments in our laboratory, placing immune spleen and solid tumor in diffusion chambers, also have revealed massive destruction of both, even when the two tissues were initially separated by a distance of several millimeters. Antibody sometimes, but not invariably, could be demonstrated in the chamber, peritoneal fluid, or serum of the host.

Close contact by the host cell may be necessary for the pickup of certain antigens, including H-2. It is known[34] that H-2 antigenic material is poorly soluble, although this may not be true for other antigens. Makari[35] and Burrows,[36] using the sensitive Schultz-Dale test, reported circulating antigen in the serum of humans with carcinoma.

Wissler and Flax[37] state that "the lymphocyte seems to be of major importance in supplying cytotoxic substances near the tumor cell" but concur with Gorer[9] that antibody may protect the lymphocyte from destruction, thus averting allergic damage to the host. From the available information, however, it is also logical to assume that lympholysis *can* have a positive value to the host. If this supposition is combined with the information given in the two preceding paragraphs, a hypothesis for explaining lymphocytic function may be derived. This suggestion can be extended to include any other type of host cell capable of lysis by antibody.

Massive (enzymatic) destruction of graft cells. Host cells, by contact with a graft or by adsorption of soluble or finely particulate graft material, may become coated with antigen from the graft. Antibody directed against graft antigens will then combine with the coated host cells and, in the presence of complement, lyse them. The ruptured host cells will then liberate their contained enzymes, which will lyse nearby cells.

This hypothesis explains many of the known observations. It depends upon, and possibly accounts for, the singular antibody sensitivity of those host cells that enter into the homograft reaction. This sensitivity is independent of cell type; variations in the cellular composition of the graft bed may be accounted for by varying chemotaxis of the graft for host cells and by the genetic composition of the host rather than by fundamental differences in rejection mechanisms. It does not depend upon the production of a special sessile antibody, but could be mediated by such an antibody, and it explains why lymphocytes are not always found in association with the graft.

The steps in this type of reaction would follow closely the generally recognized pattern of immunological response. Antigen from the graft is transported to regional lymph nodes where antibody production develops. Host cells, at first chiefly polymorphonuclear and then including other forms (depending upon the graft-host relationship) begin to infiltrate the area. Antibody circulates through the blood stream and tissue fluid in small quantities and is rapidly picked up by antigens on the graft. Host cells come into contact with graft cells and become coated with small amounts of antigen or may pick up antigen by adsorption at a distance. Opsonic or agglutinating effects of antibody may by important, since surface modifications of the graft cells by antibody could help to attract host cells. Cell-bound antibody would provide effective short-range attractive and adhesive force.

As host cells accumulate graft antigen, antibody tends to combine with the foreign antigen on their surface, thus sensitizing the altered host cells. The cell membrane of the types of cell invading the graft is known to be damaged by the activity of isoantibody and complement. The host cells rupture as complement is activated, releasing into the area the intracellular enzymes known to occur in profusion in any type of cell. Graft cells are in the close vicinity and therefore are exposed to the immediate effects of the enzymes thus liberated. Many are damaged and others are lysed.

A variant mechanism involving only localized enzymatic destruction is also possible. If sensitization of the host cell occurs only in that area directly in contact with the graft cell, local breakdown of the host cell wall will lead to rupture of the opposing segment of graft cell wall, allowing the host to digest the graft cell. This digestion may be self-limiting or may lead to fragmentation of the graft cell before digestion is complete, thus liberating particles of graft-cell antigen into the surrounding area, triggering further reactions.

Once enzymatic destruction has begun, it will become a chain reaction as antigen is liberated from the lysing tumor cells, and will be controlled by the levels of antibody and complement. Antibody will soon be exhausted in the contact zone, but should be available in the successive layers of peripheral host cells. Enzyme concentration will be greatest in the contact zone, where there will be relatively few parenchymatous cells. At least some destruction of parenchyma will occur, and blood vessels in the area should also be affected by the histaminelike substances released. If the concentration of antibody in any zone is too high, antigen will be too tightly neutralized, and anergy should prevent the reaction; if it drops too low the reaction also would be arrested. High concentrations of enzyme inhibitors might be expected shortly after the reaction, and these would lead to a refractory immunological state by inhibiting the formation of a chain reaction. Variations in local concentrations of antibody or host cells or complement or enzyme antagonists could reduce the efficiency of the response.

The hypothesis presented can be proved by determining the validity of four assumptions:

(1) Host cells can be modified in such a manner that they are lysed by isoantibody directed against the graft.

(2) Isoantibody and complement are present at the beginning of the reaction.

(3) Enzymes that can lyse cells of the graft are liberated following immunological lysis of host "immune" cells.

(4) Enzyme antagonists or inhibitory levels rise immediately after homograft rejection.

Evidence is already available upon each of these points.

(1) Evidence is presented below and in more detail elsewhere (Amos and Wakefield, to be published) to show that certain host cells do become altered and are destroyed by host antibody against the graft during the period of maximum reactivity.

(2) We know from the experience of Gorer *et al.* that antibody appears much earlier in the reaction than had been supposed from experiments using more conventional immunological methods.[6] Gorer has found low titers of antibody as early as the third day after implantation, and we have confirmed with our systems that, with this special technique, antibody can be detected during the most active phases of the rejection of L1210 by C3H mice, whereas the ordinary dextran method gives negative results at this time.

(3) Lysis by surface-active agents or distilled water is a routine procedure for the liberation of enzymes for biochemical studies. Loss of phosphate, potassium, ribonucleotides, ribonucleic acid, and protein from immunologically damaged cells has been described,[38,39] but to date release of enzyme from host cell by immune lysis has not been attempted.

(4) Burdon found a sudden drop in antitrypsin level in the first few seconds of anaphylactic shock, a rise following sublethal reactions, and a high tryptase inhibition titer in the desensitized state. He also found increased antitryptic activity in the state of anergy in tuberculosis.[40] Hardin *et al.* showed a rise in serum antitryptic level by the tenth day after homotransplantation of incompatible skin in mice where necrosis began on the eighth day.[41] When the life of the graft was prolonged by X ray or by injections of skin extract, the rise in antiprotease level was correspondingly delayed.

How specific the enzymes liberated from immune host cells might be is of course unknown. If adaptive enzymes were produced, destruction of graft cells would be maximal, but the cells must also contain many enzymes which are not specifically orientated. These also would presumably affect neighboring host cells; certainly, host parenchymatous damage and damage to vessels in the graft area is usually found. The experiments of Klein and Klein, in which a mixture of 2 tumors was grown in an F_1 animal and then transferred to a parental strain, showed that some of the compatible cells could survive a brisk homograft reaction directed at the second (carrier) population.[42] This would seem to favor specificity, but has little weight in the present argument since only limited survival was obtained with several tumors; unfortunately, these authors had no way of estimating the size of the inoculum. The question of specificity must be left unanswered at present.

Activities of host cells. The lysis of host cells by serum from the isologous strain was observed first during experiments on the cytotoxic activity of antibody on lymphoma cells grown in the peritoneal cavity and resulted from a search for evidence of sensitization of tumor cells. From the same series of experiments, it also appears that massive enzymatic destruction is only one of several graft-destructive mechanisms; phagocytosis, for example, also can be, at times, an intensely effective process.

Ascites fluids were removed from C3H mice injected intraperitoneally with

L1210 or E.L.4 ascites. A number of measurements, including total count, differential, antibody level, number of damaged cells, degree of sensitization, and susceptibility to antibody were made and will be the subject of a separate report (Amos and Wakefield, to be published).

The most striking feature was the intense histiocytic response (FIGURE 1). During the first few hours after injection polymorphonuclear cells were the predominant host cells. The histiocyte count rose rapidly to a peak of some 40 to 60 million cells. The histiocytes at this time were relatively small, showed little phagocytic activity except against red cells, and had compact cytoplasms and healthy rounded nuclei. The tumor cells multiplied rapidly at approximately the same rate as in the native strain and then suddenly began to decline (*see also* Shelton and Rice[43]). Paralleling the decline in tumor cells there was usually an abrupt fall in the number of histiocytes. The histiocytes themselves became larger, were full of irregular lipid granules usually arranged peripherally, and contained many tumor cells in all stages of digestion (FIGURES 2 to 5). As many as 20 tumor cell remnants have been counted inside 1 histiocyte. The histiocytes were distorted, often vacuolated and, at this time, very fragile. There was usually a secondary rise in histiocytic and other cells; many of the cells now found appeared more dense, and there was often more pleomorphism than in the original population (FIGURES 6 and 7). Some histocytes probably survive, as shown by gradual diminution of vacuoles and lipid droplets and progressive diminution in size, but many are lysed, and large flakes of agglutinated histiocytes are found, especially late in the reaction. Histiocyte activity is also found with other types of tumor (for example, MClM) and may be associated with extracellular digestion rather than phagocytosis (FIGURES 8 and 9).

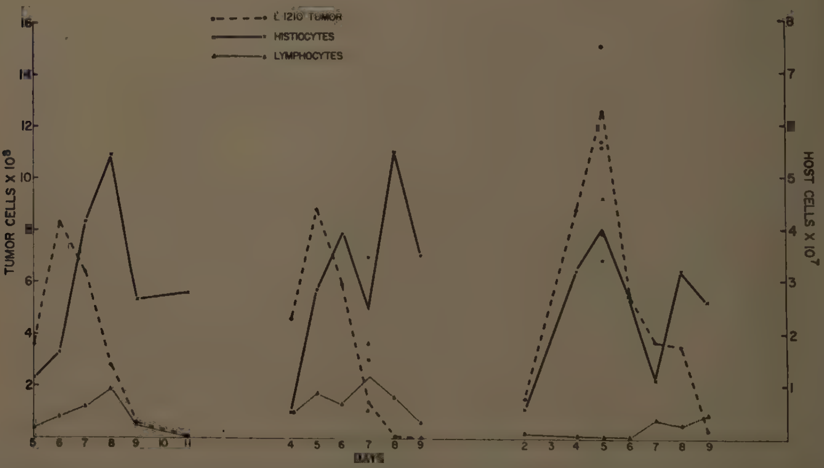

FIGURE 1. Growth curves of L1210 ascites and host cellular response of C3H mice, following inoculation of 20 million lymphoma cells.

Phagocytosis must account for the disappearance of very many tumor cells. Examination of histiocytes at the time of the intensive stage of phagocytosis

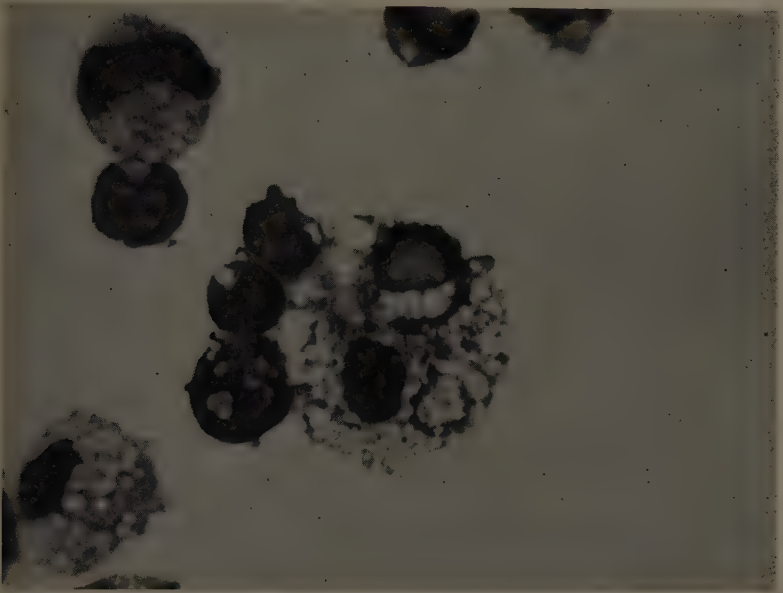

FIGURE 2. C3H histiocyte beginning digestion of recently ingested E.L.4 cell. At least 1 of the other 3 cells closely attached appears to be in the early stages of ingestion. ×600.

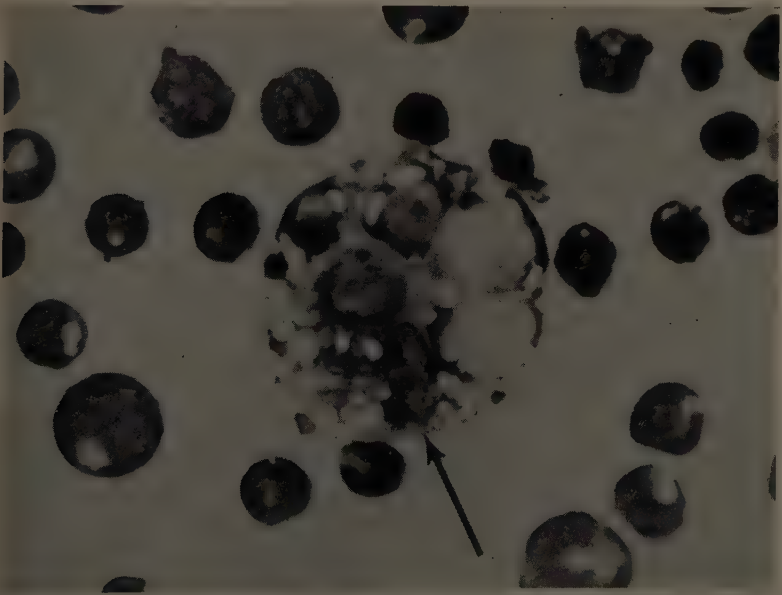

FIGURE 3. Actively phagocytic C3H histiocytes 8 days after the injection of L1210 ascites. The arrow points to histiocyte nucleus. ×600. (*See also* FIGURES 4 and 5.)

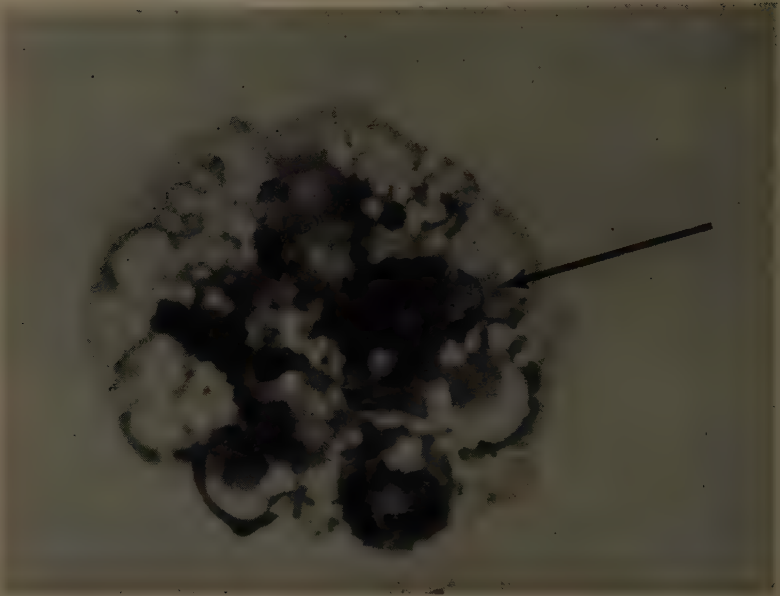

FIGURE 4. Actively phagocytic C3H histiocytes 8 days after the injection of L1210 ascites The arrow points to histiocyte nucleus. ×600. (*See also* FIGURES 3 and 5.)

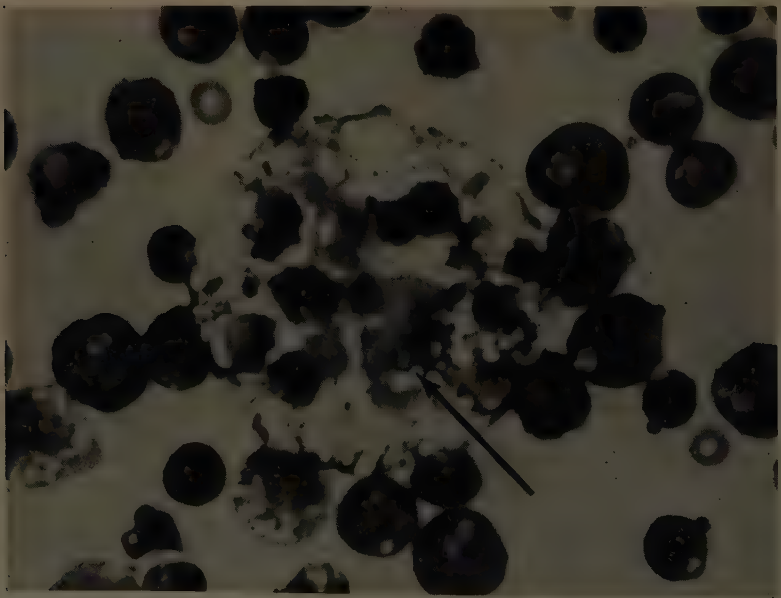

FIGURE 5. Actively phagocytic C3H histiocytes 8 days after the injection of L1210 ascites. The arrow points to histiocyte nucleus. ×600. (*See also* FIGURES 3 and 4.)

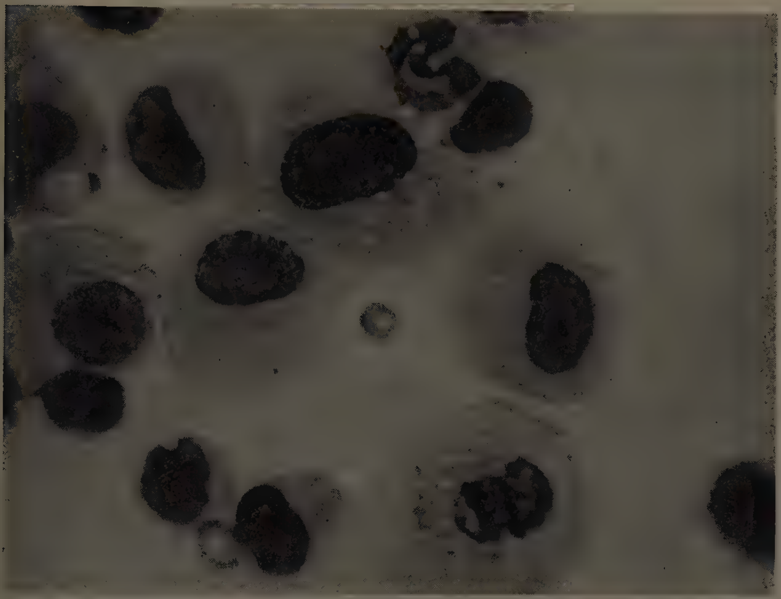

FIGURE 6. C3H histiocytes, mononuclear cells, and eosinophil in late stage of rejection of E.L.4 ascites. ×600.

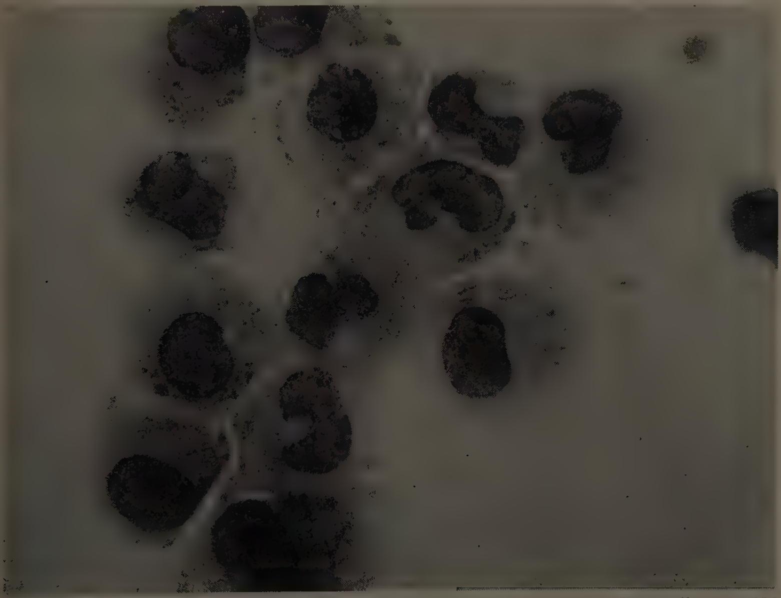

FIGURE 7. C3H histiocytes after rejection of 20 million L1210 ascites cells. ×600.

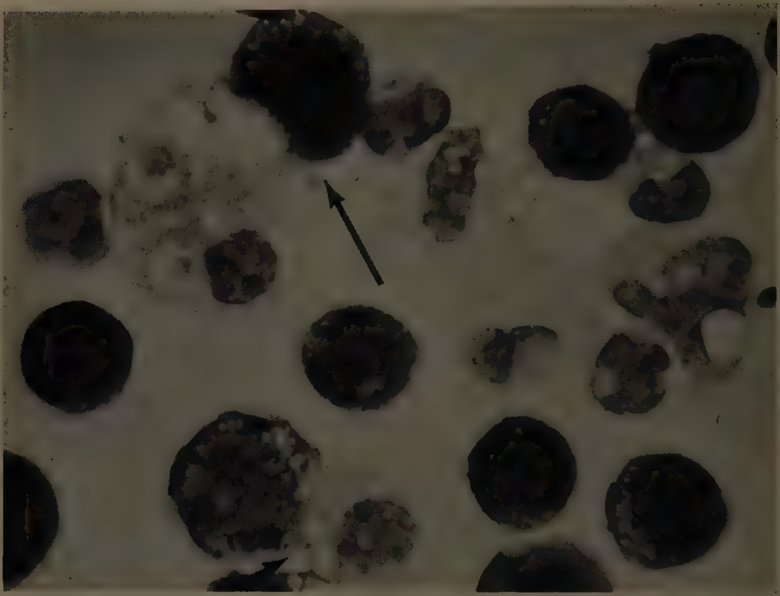

FIGURE 8. The response of 129 mice to MClM ascites. Note the loss of resolution of the cytoplasmic boundary of the tumor cell attached to the smaller histiocyte, below the pyknosis of the tumor cell, and the large opposing vacuole in the larger histiocyte (*arrow*). ×600.

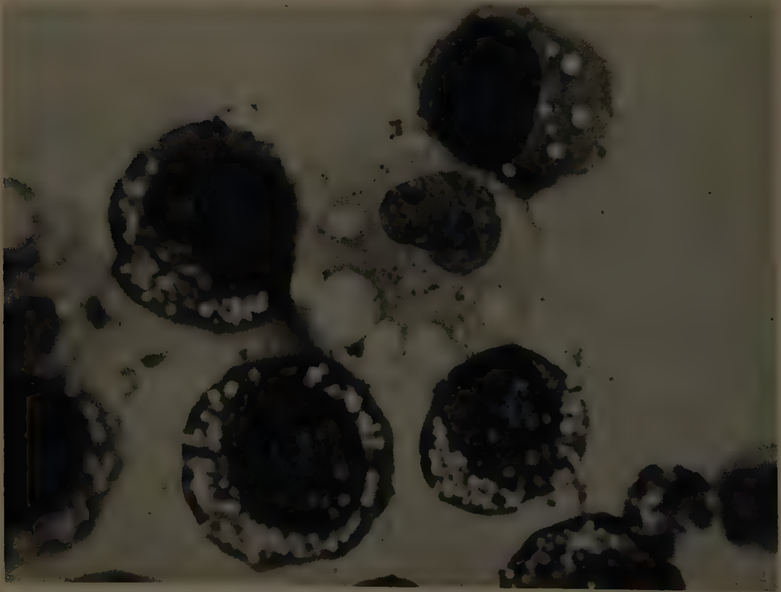

FIGURE 9. MClM ascites in C58 mice. Extensive vacuolation of histiocyte cytoplasm. Three of the nuclei are crowded toward the histiocyte. There is no attempt at phagocytosis. ×600.

indicates an average of up to 2 ingested cells per histiocyte. For a count of 40 million histiocytes this would indicate destruction of 80 million cells at any one time, excluding any activity of fixed phagocytes. Many of the cells not obviously active had intensely vacuolated cytoplasms, and electron microscopy indicates the presence of digested cell remnants and masses of lipid droplets in these cells (FIGURE 10). Since the onset of intense phagocytosis is sudden and many cells have proceeded to complete destruction, it is probable that digestion is rapid and that the cells actually taken up greatly exceed this number.

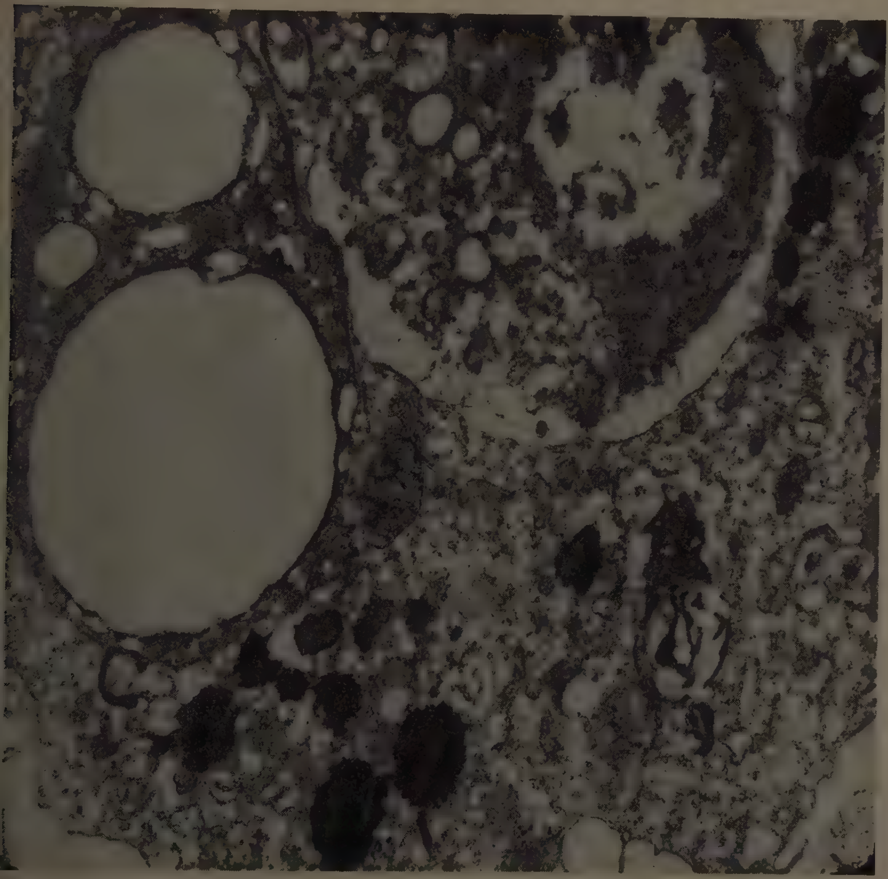

FIGURE 10. C3H histiocyte, showing L1210 ascites cells in varying stages of digestion. The numerous scattered lipid droplets are commonly found in this combination. ×20,000. Ultramicrograph by courtesy of Leon Journey.

Snell has commented on the paucity of information concerning phagocytosis of tumor cells, and he believes that this process accounts for the removal of dead cells.[44] Similar opinions were also expressed by Koprowska[45] and by Algire.[46] It is difficult to disprove this view, but trypan-blue staining of fresh ascites during active rejection usually reveals very few damaged tumor cells. The scavenging efficiency would necessarily be very high to remove damaged cells so rapidly. Although digestion appears to be rapid, occassional cells are seen in the process of ingestion. These cells appear morphologically normal. Unstained cells have been seen inside a histiocyte that had been stained by specific isoantibody. Phagocytosis of L #1 cells in mitosis and other morphologically intact cells has been clearly shown by Shelton and Dalton[47] and has been reported with the DBA/2 thymoma in C57BL mice by Weaver.[33] It appears extremely likely that phagocytosis can be an active destructive mechanism and not merely a scavenging activity.

Antigenic alteration of host cells. Complicated antigenic changes that have a bearing on the remote-digestive hypothesis previously advanced (page 277) are observed.

Histiocytes from C3H mice injected with L1210 or with E.L.4 at a certain critical stage in tumor rejection are damaged by isoantibody directed against either the host or the tumor, even in the absence of added heterologous complement.

In experiments in which L1210 was used, the antibodies were DBA/2-anti-C3H, and C3H-anti-DBA/2; with E.L.4, C57-anti-C3H, and C3H-anti-C57. The results were similar with each system and have been repeated several times, but they are most clearly obtained when histiocytes first make up the bulk of the population. Within 24 hours the unusual histiocytes either have been destroyed by the host's own antibody or else have eliminated or digested the tumor antigen. At an earlier stage, if the reaction occurs, it is obscured by the high concentration of tumor cells.

Normal host cells, including histiocytes at a later stage, are never affected *in vitro* by isoantibody against the tumor even in the presence of added complement. Cytotoxic effects have not been seen with mouse isoantibodies unless heterologous complement was added, and isoantibody against the host has no effect by itself on host histiocytes. Mouse complement had little if any effect on cells sensitized *in vitro* and minimal but highly characteristic effects on cells sensitized *in vivo* in diffusion chambers.

These results imply modification of the surface of the host histiocyte by tumor cell antigen or incorporation of tumor antigen into the histiocyte.

The addition of isoantibody directed against the host to the histiocyte-rich ascites population at about the time when only some 10 per cent of tumor cells remain causes rapid lysis of up to 75 per cent of the histiocytes as compared to the controls in Ringer's solution or guinea pig serum and a staining upon addition of trypan blue of up to 50 per cent of the remaining cells. Many of the histiocytes show the same staining and lysis upon addition of antibody against the tumor. The addition of guinea pig complement to this reaction has little effect. Added to the antibody against the host, it causes destruction of the majority of the remaining cells; the undamaged cells are probably, but not certainly, tumor cells.

At first it appeared that histiocytes might be secretors of complement or might be vitally stained by trypan blue, but an attempt to repeat the experiment the following day was unsuccessful, and the anomalous result could not be obtained again until the entire experiment was repeated. Again the effect was transient, occurring when approximately the same stage of tumor rejection was reached. To date 9 mice of 33 tested have shown lysis of host cells by antibody against the graft; the other 24 have not. These 9 were all in the stage of active vegetation.

Histiocytes digest L1210 and E.L.4 cells and to a lesser extent, MClM and TA3 cells. According to the clear evidence of Shelton and Dalton,[47] they do not digest L #1 cells, although they can ingest them. There are probably differences in antibody level in the different hosts and also in the availability of antigen on the cell surfaces. L #1 is feebly antigenic[48] and, although high-titered antibodies can be formed in C57BL mice after repeated injections, they tend to appear very late. The dependence of histiocyte activity upon opsonic or other activities of antibody has not yet been demonstrated.

Histiocytes are not the only cells found in the ascites, and the number and relative proportions of host-cell types found differ considerably with various tumor-host combinations.

The injected cell dose may be critical in determining the type of cellular response. On occasion eosinophils, neutrophils, and lymphocytes may be common. Only certain tumor cells appear to be as susceptible to histiocyte digestive phagocytosis as L1210.

Phagocytosis is not always found to accompany a histiocytic response and is perhaps rather limited in its effect on solid transplants. Closely bound clumps of histiocytes and tumor cells are found with some tumor-host combinations. These are seen in both smears and electron-microscopic preparations. The tumor cell is condensed and flattened toward the surface in contact with the histiocyte. Vacuoles are seen in a corresponding segment of the host cell and sometimes the cytoplasms appear to have fused (FIGURES 8 and 9). These changes do not appear to be the result of chance contact.

Antibody formation in mice so frequently follows transplantation of histoincompatible tissue that it must be considered as an integral part of the rejection mechanism. The simplest hypothesis combining available information on the variation in host cell types at the graft site, the peculiar sensitivity of the host-induced stroma to immune mechanisms, the resistance of many somatic cells to isoantibody, and the immunological changes found in the host cells is that the isoantibodies act not only on the graft cells but upon modified host cells in the vicinity of the graft, and that enzymes released from host cells then react with the graft. It is interesting that Kaliss[49] reported that, although a host inflammatory response occurred in both pretreated and normal animals, the host cellular response diminished in the later stages of enhancement. This and other phenomena need investigation before the general applicability of the hypothesis can be assumed, but at present most of the exceptional cases reported can be incorporated readily. Experimentation on possible enzymes liberated in the region of the graft and determination of their specificity is urgently needed.

Summary

TABLE 1 summarizes the mechanisms that may be active in homograft rejection.

Direct lysis is probable when the graft is susceptible to the action of antibody and complement, when the antibody level is high, and when complement is freely available.

Phagocytosis appears important in certain situations such as those described when E.L.4 or L1210 is injected into C3H mice, but may be of less importance with other tumors and in other strains. Both of these mechanisms can lead to hemolytic anemia and jaundice, since red cells as well as other types of host cell can become coated with antigen and therefore ingested or lysed.

Massive enzymatic destruction is suggested as an explanation of some of the observations of Algire.[19,20] Host cells become coated with antigen from the graft, and an antibody-antigen-complement reaction accordingly takes place on the surface of the affected host cell. The host cell lyses, releasing intracellular proteolytic and oxidative enzymes that kill and lyse other cells, including those of the graft. These in turn release fresh antigen which perpetuates the reaction.

Direct enzymatic destruction is a variant of, and possibly a primer for, the above reaction. The mechanism is essentially the same, but it occurs in cells in intimate contact and is limited to the area of contact. This reaction is the one most likely to be mediated by cell-bound antibodies. Feebly antigenic cells will be resistant, since the reaction is less likely to occur in the absence of adequate amounts of antigen.

TABLE 1
MECHANISMS OF GRAFT REJECTION

Participant	Mode of action	Concomitant effects
Antibody + complement	Direct lysis	Anemia, jaundice
Histiocytes	Phagocytosis	
"Immune" cells	Direct (enzymatic)	
Host cell + antibody + complement	Remote (enzymatic)	Host parenchyma damage
Vascular Stromal	Ischemia, fibrosis	

Remote enzymatic damage may result in damage to any cell in the vicinity, both graft and host, including host parenchyma and stroma, whereas direct enzymatic damage will be more limited in effect.

Vascular changes may follow directly upon the release of toxic materials such as histamine in the vicinity of the graft, or they could be the result of an allergic reaction occurring on the vessel wall.

References

1. GORER, P. A. 1937. Genetic and antigenic basis of tumour transplantation. J. Pathol. Bacteriol. **44:** 691.
2. GORER, P. A. 1942. The role of antibodies in immunity to transplanted leukaemia in mice. J. Pathol. Bacteriol. **54:** 51.
3. GORER, P. A. & D. B. AMOS. 1956. Passive immunity in mice against C57BL leukosis E.L.4 by means of iso-immune serum. Cancer Research. **16:** 338.
4. GORER, P. A. & P. O'GORMAN. 1956. The cytotoxic activity of isoantibodies in mice. Transplantation Bull. **3:** 142.
5. GORER, P. A. & Z. MIKULSKA. 1954. The antibody response to tumor inoculation. Improved methods of antibody detection. Cancer Research. **14:** 651.

6. Gorer, P. A., Z. B. Mikulska & P. O'Gorman. 1959. The time of appearance of isoantibodies during the homograft response to mouse tumors. Immunology. **2:** 211.
7. Mitchison, N. A. 1955. Studies on the immunological response to foreign tumor transplants in the mouse. I. The role of lymph node cells in conferring immunity by adoptive transfer. J. Exptl. Med. **102:** 157.
8. Winn, H. 1960. J. Natl. Cancer Inst. In press.
9. Gorer, P. A. 1956. Some recent work on tumor immunity. Advances in Cancer Research. **4:** 149.
10. Lumsden, T. 1931. Tumor immunity. Am. J. Cancer. **15:** 563.
11. Lumsden, T. 1937. Cytotoxins lethal to nucleated mammalian cells. Am. J. Cancer. **31:** 430.
12. Moppett, W. 1935. A review of Lumsden's work on tumor immunity. J. Cancer Research Committee Univ. Sydney. **7:** 91.
13. Ludford, R. J. 1934. Factors influencing the growth of normal and malignant cells in fluid culture media. Proc. Roy. Soc. London. **B115:** 278.
14. Phelps, H. J. 1937. A note on the non-specific action of so-called "anti-cancer serum." Am. J. Cancer. **31:** 441.
15. Amos, D. B. 1959. Some iso-antigenic systems of the mouse. Proc. 3rd Canadian Cancer Conf. **3:** 241.
16. Amos, D. B., P. A. Gorer & Z. Mikulska. 1955. An analysis of an antigenic system in the mouse (the H-2 system). Proc. Roy. Soc. London. **B144:** 369.
17. Hoecker, G. 1956. Genetic mechanisms in tissue transplantation in the mouse. Cold Spring Harbor Symposia Quant. Biol. **21:** 355.
18. Amos, D. B. & J. D. Wakefield. 1958. Growth of mouse ascites tumor cells in diffusion chambers. I. Studies on growth rate of cells and of the rate of entry of antibody. J. Natl. Cancer Inst. **21:** 657.
19. Algire, G. H. 1957. Diffusion-chamber techniques for studies of cellular immunity. Ann. N. Y. Acad. Sci. **69**(4): 663.
20. Algire, G. H., J. M. Weaver & R. T. Prehn. 1956. Studies on tissue homotransplantation in mice using diffusion chamber technique. Ann. N. Y. Acad. Sci. **64**(5): 1009.
21. Amos, D. B. & J. D. Wakefield. 1959. Growth of mouse ascites tumor cells in diffusion chambers. II. Lysis and growth inhibition by diffusible isoantibody. J. Natl. Cancer Inst. **22:** 1077.
22. Amos, D. B. & J. D. Wakefield. 1959. Potentiation by normal serum of the cytolytic activity of iso-antibody. Proc. Am. Assoc. Cancer Research. **2:** 3.
23. Levy, A. L. 1958. Complement levels in mammals. Proc. Soc. Exptl. Biol. Med. **99:** 584.
24. Gorer, P. A. 1956. The value of ascites tumors in problems of tumor immunity. Ann. N. Y. Acad. Sci. **63**(5): 882.
25. Gorer, P. A. & E. A. Boyse. 1959. Some reactions observed with transplanted reticulo-endothelial cells in mice. Proc. Colloquium on Biological Aspects of Transplantation, Liége. **12:** 193.
26. Loeb, L. 1945. *In* The Biological Basis of Individuality. 711 pp. Thomas. Springfield, Ill. 1947 (second printing).
27. Woglom, W. H. 1929. Immunity to transplantable tumours. Cancer Rev. **4:** 129.
28. Dreisbach, M. E., G. D. Snell & R. S. Speirs. 1956. Quantitative studies of the local cellular response to isoantigens. J. Natl. Cancer Inst. **17:** 297.
29. Murphy, J. B. & J. J. Morton. 1915. The lymphocyte as a factor in natural and induced resistance to transplanted cancer. Proc. Natl. Acad. Sci. **1:** 435.
30. Brent, L., J. Brown & P. B. Medawar. 1958. Skin transplantation immunity in relation to hypersensitivity. Lancet. **2:** 561.
31. Medawar, P. B. 1958. The immunology of transplantation. Harvey Lectures, Ser. **51:** 144.
32. Weaver, J. M., G. H. Algire & R. T. Prehn. 1955. The growth of cells *in vivo* in diffusion chambers. II. The role of cells in the destruction of homografts in mice. J. Natl. Cancer Inst. **15:** 1737.
33. Weaver, J. M. 1958. Destruction of mouse ascites tumor cells *in vivo* and *in vitro* by homologous macrophages, lymphocytes and cell free antibodies. Proc. Am. Assoc. Cancer Research. **2:** 354.
34. Kandutsch, A. A. 1957. Studies on a substance that promotes tumor homograft survival (the "enhancing substance"). J. Exptl. Med. **105:** 125.
35. Makari, J. G. 1955. Use of Schultz-Dale test for detection of specific antigen in sera of patients with carcinoma. Brit. Med. J. **2:** 1291.
36. Burrows, D. 1958. Schultz-Dale test for detection of specific antigen in sera of patients with carcinoma. Brit. Med. J. **1:** 368.

37. Wissler, R. W. & M. H. Flax. 1957. Cytotoxic effects of anti-tumor serum. Ann. N. Y. Acad. Sci. **69**(4): 773.
38. Ellem, K. O. 1958. Some aspects of the ascites tumor cell response to a heterologous antiserum. Cancer Research. **18:** 1179.
39. Green H., R. A. Fleischer, P. Barrow & B. Goldberg. 1959. The cytotoxic activity of immune gamma globulin and complement on Krebs ascites tumor cells. J. Exptl. Med. **109:** 511.
40. Burdon, K. L. 1949. Evidence for the controlling influence in protease inhibitors in allergic phenomena. Federation Proc. **8:** 401.
41. Hardin, C. A., A. A. Werder, W. D. Hoofer & M. S. Liggett. 1957. The theory of protease activation and its role in the rejection of homotransplants. Surgery. **41:** 752.
42. Klein, E. & G. Klein. 1954. Differential survival of solid tumor cells after inoculation into established ascites tumors. Cancer Research. **14:** 139.
43. Shelton, E. & M. E. Rice. 1958. Studies on mouse lymphomas. III. Behaviour of tumor—and non-tumor—cell populations during growth of three ascites lymphomas. J. Natl. Cancer Inst. **21:** 163.
44. Snell G. D. 1957. The homograft reaction. Ann. Rev. Microbiol. **11:** 439.
45. Koprowska, I. 1956. Exfoliative cytology in the study of ascites cells. Ann. N. Y. Acad. Sci. **63**(6): 738.
46. Algire, G. H. 1956. Discussion. Ann. N. Y. Acad. Sci. **64**(5): 1026.
47. Shelton, E. & A. J. Dalton. 1959. Electron microscopy of emperipolesis. J. Biophys. Biochem. Cytol. **6:** 513.
48. Amos, D. B. 1956. Serological differences between comparable diploid and tetraploid lines of three mouse ascites tumors. Ann. N. Y. Acad. Sci. **63**(6): 706.
49. Kaliss, N. 1955. Induced alteration of the normal host-graft relationship in homotransplantation of mouse tumors. Ann. N. Y. Acad. Sci. **59**(3): 385.

Discussion of the Paper

Peter A. Gorer: A few years ago, when these monographs on tissue homotransplantation began to appear, one was asked what was the relationship between homograft immunity and classic immunity. We knew that antibodies could be lytic to either bacteria or red cells in a test tube, and we knew they could do certain things *in vivo*, but the sort of dogma that I was brought up on was that the real function of antibodies was to act in a cooperative fashion with the cellular defenses of the host.

In that sense it does seem that homograft immunity is not entirely classic. Whether it is classic seems to depend upon the nature of the target cell, whether the target cell is inherently susceptible to the cytotoxic activity of an antibody, whether it is not inherently resistant, or whether an anatomical situation makes it unavailable.

We have done most of our work with ascites tumors, where the question of availability does not enter. One can divide transplantable tumors into three classes. We have Sarcoma 1, which was used by Mitchison and has been used extensively by Kaliss. In so far as we know, this tumor is completely resistant to the cytotoxic activity of antibody; for a long time, that was considered the homograft reaction. Anything true of Sarcoma 1 was assumed to be true of everything else.

At the other extreme, No. 2, we have the leukemias, concerning which much is said in these pages. With the leukemias, we include things such as lymph node cells, dealt with by the Harrises and, to some extent, by Siskind and Thomas, and bone marrow cells, all of which are susceptible both *in vitro* and *in vivo.*

The effect of antibody *in vivo* in that type of cell is to kill or at least to inhibit. In the case of a highly resistant cell, this peculiar phenomenon of enhancement is obtained, in which case a graft survives in a host where it would not do so if the host had not been pretreated.

The difficult ones, constituting the third class, are the intermediate type of tumors that consist of mixed populations of cells. Kaliss and I have shown that one could obtain almost any sort of results, depending on the experimental conditions: broadly speaking, the larger the dose of serum, the more inhibition; with smaller doses of serum, one tended to obtain enhancement.

Recently we have tested a number of other tumors. Without going into experimental details, we find that BPH is a very representative tumor in so far as mammary tumors and carcinomas are concerned.

We then come to the question of why one should obtain both results and, if you like, what is the nature of enhancement, since enhancement is the unclassic aspect of transplantation immunity.

Many of us have unburdened ourselves of theories of enhancement. I plead guilty; Medawar has done it; Snell has done it; and Kaliss has done it. I think I can speak for all of us if I say that none of us is really very devoted to any of the theories we have put forward, but all of us, I think, would agree on one thing: that somehow humoral antibody can interfere with the proper function of the host cells.

We have begun to use a technique devised by Henry Winn of Bar Harbor, Me. In principle, it is a very simple technique. One takes immune lymph node cells and mixes them with the target (in this case, the tumor) and injects the two together. One can obtain inhibition under proper conditions, or one may establish such conditions that the cells do nothing. One may use a tumor such as BPH and adjust the serum dose so that it enhances; then one can find what happens if both are used together. It is unquestionable as regards BPH that, if an ineffective dose of cells and an enhancing dose of serum are used together, then one obtains almost complete inhibition. In that case, there is evidently a synergic action between humoral antibody and immune cells. This is just the beginning and, in fact, it does not result in the way that we want it. What we are trying to ascertain is under what conditions one will obtain synergy and under what conditions one will obtain antagonism.

Toolan: Since Amos raised the possibility that there might be a sensitization of the tumor cell or the tissue to be destroyed, I mention some things that have been puzzling us for some time.

In a series of more than 150 rabbit homografts whereby we demonstrated the slow rejection phenomena by discontinuation of cortisone, one of the things that puzzled us exceedingly was the fact that after we had first seen hyalinization, which occurred from the first week after the discontinuation of the cortisone, we then saw an influx of polymorphs that adhered to the base of the epidermis for a period of four to five days in long linear arrangements. These finally migrated through the epidermis and were discharged.

We were not able to decide what role these polymorphs played, but it did seem that together they might have the role of a sensitizer because it was after they had already been present that the lymphocytes then appeared and brought about a tremendous lysis of the graft's epidermal cells.

TILLETT: I ask Stetson what is perhaps an oversimplified question. A primary homograft is placed upon a rabbit. Subsequently, a second homograft is placed upon the same rabbit, which he rejects. Are cytotoxic antibodies present in the serum of that rabbit at the time he rejects the second homograft?

STETSON: Thus far we have not been able to detect cytotoxic antibodies in rabbit serum. There are certainly hemagglutination antibodies present, but as yet no satisfactory test for cytotoxic activity has been devised for this species.

MERRILL: In regard to the demonstration of cytotoxic antibody, our experience has been that it is probably not so much the number of grafts or the graft dosage, but the technique that is used. We had thought in our initial experiments that perhaps the grafts were not sufficiently large and that the interval might have something to do with it, but I think the technique for demonstrating it probably is the clue to it rather than the graft size or the number of grafts.

We have used two grafts in our studies and have attempted to demonstrate this four or five days after we were sure that the second graft had been rejected in order that we might get a maximum titer, but I am not sure that that made all the difference. I think it was probably the technique.

SERUM ANTIBODIES FOLLOWING HOMOTRANSPLANTATION OF HUMAN CANCER CELLS*

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Previous publications have recorded that, in patients with advanced neoplastic disease, the rejection of homotransplants of human cancer cell lines is impaired[8] and that, when rejection has been accomplished in either normal or sick recipients, an immunity to further implantation exists, as indicated by accelerated rejection of repeat implants of the same or other cancer cell lines.[7] Antibodies were not detected in serum from these recipients by complement-fixing, precipitin, or neutralization techniques.[7] This paper reports the demonstration of serum antibodies in these same recipients by use of the more sensitive tanned erythrocyte hemmagglutination technique.

Methods

Methods of homotransplantation and evaluation of transplant growth have been described in previous papers.[7,8] The antibody detection technique is basically that described by Boyden,[1] but several modifications have been made. The cell lines studied were Toolan's HEp 1, HEp 2, and HEp 3,[5] Osgood's J-111,[4] and Amnion B (a human amnion cell line established in this laboratory and maintained about 6 months when used in these studies).

Test antigens. Human cancer cell lines grown on glass without clot in tissue culture were harvested by trypsinization (0.05 or 0.5 per cent), and washed 2 or 3 times in about 500 volumes of Gey's salt solution† and then once in unbuffered isotonic saline. Only freshly harvested cells were used for the antigen extractions. Water in all solutions used for antigen preparation or serologic testing was once-distilled and deionized with a Deeminac ion exchange resin filter‡ and plastic squeeze bottle. The cells were resuspended in 2 volumes of chilled (4° C.) *p*H 7.2 phosphate-buffered isotonic saline[11] containing 10.0 mg./l. tetrasodium ethylenediamine tetraacetate (EDTA) and homogenized for several minutes in a chilled glass-and-Teflon Potter-Elvehjem type homogenizer immersed in an ice-water bath. This suspension was centrifuged at 1000 g for 10 min. in a refrigerated PR2 centrifuge§ with horizontal head (2000 rpm). After the turbid supernatant was harvested, the sediment was again extracted by same process, and this sediment was discarded. The pooled supernatants were diluted further with 4 volumes of *p*H 7.2 phosphate-buffered saline and centrifuged at 7000 g in the offset head of the same centrifuge for 30 min. The sediment was discarded. The slightly opalescent supernatant was harvested. These "stock antigens" were diluted further to either 1:100 or 1:200 (depending upon visual estimate of turbidity) in *p*H 6.4 phosphate buffered isotonic saline[11] containing 10.0 mg./l. EDTA.

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* The work described in this paper was supported in part by grants from the National Cancer Institute, Public Health Service, Bethesda, Md.

† Microbiological Associates, Washington, D. C.

‡ Crystal Research Laboratories, Hartford, Conn.

§ International Equipment Co., Boston, Mass.

Nitrogen contents (micro-Kjeldahl method) of 7 of these "stock antigens" which gave good hemagglutination tests ranged between 0.5 to 2.3 mg./ml. Thus final nitrogen concentrations during the erythrocyte coating step (*see below*) were 2.7 to 21.0 γ/ml.

Erythrocyte preparation. Tannic acid was Mallinckrodt analytical reagent. The 1:1000 stock solution in water was mixed with Dowex 1-X4 anion exchange resin* to remove contaminating zinc. The resin had been washed twice in deionized distilled water and once in absolute ethanol, and dried. The amount needed was computed on basis of the stated zinc content of the tannic acid and the exchange capacity of the resin, and a slight excess was used. After 1 to 2 hours at room temperature, the resin was removed by centrifugation. This procedure appeared to diminish nonspecific agglutination and lysis of the tanned erythrocytes.

The aqueous tannic acid solution was then diluted further to 1:20,000 using *p*H 7.2 saline, mixed with an equal volume of 2.5 per cent sheep red blood cells (SRBC) in *p*H 7.2 EDTA-saline, and incubated in a 37° C. water bath for 10 min. After washing twice with the buffered saline, a 2.5 per cent suspension of these "tanned" SRBC was made in unbuffered 0.15 *M* saline. Then two volumes of the test antigen preparation (*see above*) in *p*H 6.4 saline were mixed with 1 volume of the tanned SRBC suspension and incubated at room temperature for 30 min.

These antigen-coated SRBC were then washed twice with unbuffered 0.15 *M* saline containing 0.5 per cent normal rabbit serum (NRS) inactivated and absorbed with washed SRBC in the same way as the test sera (*see below*) and finally resuspended in NRS-saline at 2.5 per cent.

Antisera. All of the sera from normal homograft recipients and most of the sera from cancer patients had been stored at −70° C. for 2 months to 3 years prior to testing. All sera were inactivated at 56° C. for 30 min. and absorbed for at least 1 hour at room temperature with an equal volume of washed and packed SRBC before the test.

Rabbit antihuman cancer cell sera (supplied by Leonhard Korngold then of this institute) were included in many tests as a positive control. These were inactivated and adsorbed as above with SRBC and pooled human red blood cells (containing A, B, and Rh agglutinogens) and pooled lyophilized human plasma.

Hemagglutination test. Five-tenths cc. volumes of serial twofold dilutions of the sera being tested were distributed into round-bottom Kahn tubes (10 × 75 mm.) by the usual pipetting procedures. Each test included antigen controls (no serum), serum controls (no antigen), and a "known positive" control (either rabbit or human serum). See FIGURE 1.

One drop (0.05 ml.) of antigen-coated SRBC suspension was then added to each tube (except the serum-control tubes, which received tanned but noncoated SRBC).

The racks of tubes were thoroughly shaken and then incubated at room temperature (21 to 29° C.) for 3 to 4 hours. The tests could usually be read at this time, but incubation was routinely continued in a refrigerator (4° C.) over-

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* Baker Chemical Co., Phillipsburg, N. J.

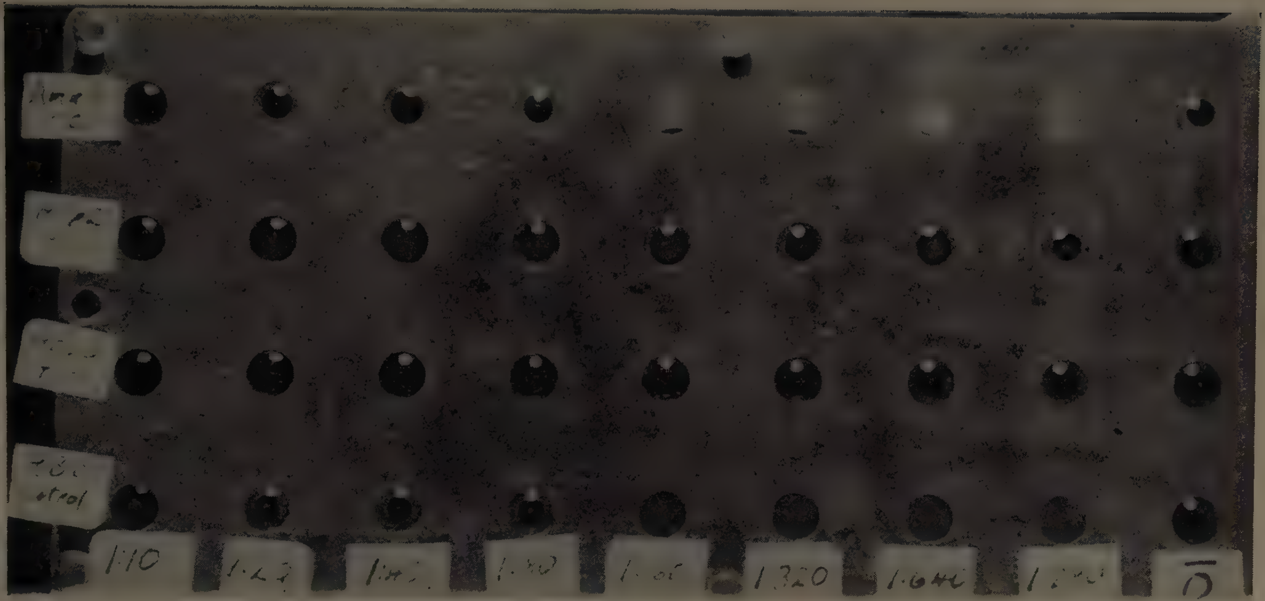


FIGURE 1. Sedimentation patterns in hemagglutination test with serum of a normal recipient four weeks after homotransplantation. Labels indicate type of antigen and serum dilutions. No agglutination of tanned sheep erythrocytes without antigen (*bottom row*) or coated with Amnion B cell antigen (*top row*). Positive two plus agglutination patterns with HEp 2 and HEp 3 antigens through 1:20, one plus reaction with HEp 2 to 1:160, and with HEp 3 to 1:80. Higher dilutions plus-minus or negative.

night, as recommended by Boyden. The incubation period at room temperature was shortened occasionally.

The erythrocyte sedimentation pattern was read[9] after overnight incubation in refrigerator. The tubes were then thoroughly shaken and were read again after standing at room temperature for another 3 to 4 hours. The two readings almost always gave the same results. The only discrepancies were unstable sedimentation patterns suggestive of agglutination; these were seen in occasional tests in some tubes immediately after removal from the refrigerator.

Intensity of agglutination from negative to ++++ was graded by Stavitsky's criteria. Rabbit antihuman cancer cell sera gave strong agglutination reactions, including Stavitsky's +++ and ++++, and had end points of 1:12,800 or more. The human antihuman cancer cell sera never caused +++ or ++++ agglutination, but gave clear ++ and + patterns (FIGURE 1). The last tube showing a one plus reaction was taken as the end point.

Results

Antibodies in normal recipients. Circulating antibody was demonstrated with one or more antigens in all but 2 of the 37 normal recipients of cancer cell lines studied to date. Antibody was detectable by day 14 after implantation, and with 2 exceptions was still present at the same or higher levels on day 28. Only 2 normal recipients still had detectable antibody (both at 1:10) at 6 weeks. Maximum titer after a primary implant was 1:80 and, in 2 recipients, maximum was only 1:10. These data are summarized in TABLE 1 and FIGURE 2.

Normal recipients of implants of a single cell type (HEp 2 or HEp 3) showed cross reactions with each of the four cancer cell lines studied to date (HEp 1, HEp 2, HEp 3, and J-111). See TABLE 1 and FIGURE 3. Mean antibody titers were slightly greater against antigens of the same cell type that was implanted, but the differences were slight, and individuals showed considerable overlap, so the differences may not be significant.

Men who received simultaneous implants of both HEp 2 and HEp 3 showed a slightly greater mean antibody response (both higher and longer) than those who received only one of these cell types, (FIGURE 4) but again the differences cannot be accepted as significant since the number in each category studied by serial serum specimens was small (3 to 10) and there was considerable overlap among individuals.

Among 19 normal recipients who had repeated implants (homotransplants on 2, 3, or 4 occasions at intervals of 3 to 15 months) and whose sera were studied at appropriate intervals, 9 had detectable serum antibody at 1 week and/or at 6 to 7 weeks whereas, among 27 individuals so studied after primary implants, only 3 had detectable antibody at these times. Maximum titers at 2, 3, or 4 weeks were 1:80 or higher in 8 of the 20 recipients of repeat implants, whereas only 6 of 31 recipients of primary implants had titers in excess of 1:40. These results are interpreted as a booster response to repeated homotransplants. It is noteworthy, however, that not all recipients showed such evidence of a booster response and 1 normal recipient had no detectable serum antibodies even after 3 successive implants. These data are illustrated in FIGURE 5, which compares primary recipients with a simultaneously inoculated group of men who had previously received live cells of the same and other cell types on 2 or 3 previous occasions at intervals of 5 to 15 months, and in FIGURE 6, which illustrates that this booster-type antibody response also occurred after live cancer cell implants when only killed cells had been used for the primary implant. After inoculation of 6 normal recipients with killed HEp 2 or killed HEp 3 cells as a primary implant, only 1 had detectable antibodies, and this one was positive only on day 14 at 1:10. However, after challenge with both HEp 2 and HEp 3 live cancer cells, these 6 individuals all showed a booster-

TABLE 1

DETECTION OF SERUM ANTIBODY AFTER PRIMARY AND REPEAT HOMOTRANSPLANTS OF HUMAN CANCER CELL LINES IN NORMAL RECIPIENTS

Figures Indicate Number of Individuals

Cell line for primary implant	Test antigens	Antibody after primary implant			Antibody after repeat implant (1)		
		Yes	No	Remarks	Yes	No	Remarks
J-111	J-111	4	0		1	0	
HEp 1	J-111	—	—		1	0	
	HEp 2	1	0		1	0	
HEp 2	HEp 1	3	0		—	—	
	HEp 2	5	1	(2)	1	1	(2)
	HEp 3	4	0		1	0	
	J-111	2	0		1	0	
HEp 3	HEp 1	3	0		1	0	
	HEp 2	5	1	(3)	3	1	(3)
	HEp 3	3	0		2	0	
HEp 3 and HEp 2	HEp 1	6	0		2	0	
	HEp 2	19	1	(2)	6	0	
	HEp 3	8	0		—	—	
	J-111	5	0		4	1	(4)
Total: live cancer cell recipients		35	2	(5)	13	1	(5)
Amnion and fibroblasts	HEp 2	0	2		1	0	
	HEp 3	0	2		1	0	
	Amnion B	0	2		0	1	
Killed HEp 2	HEp 2	0	3		3	0	
	HEp 3	0	3		3	0	
	Amnion B	0	1		0	3	
Killed HEp 3	HEp 2	0	3		3	0	
	HEp 3	1	2		3	0	
	Amnion B	—	—		0	3	

Remarks: (1) Repeat implants were HEp 2 plus HEp 3 in all persons studied; (2) nonreactor against HEp 2 antigen was not tested against other antigens; (3) nonreactor against HEp 2 antigen was positive with HEp 3 antigen; (4) nonreactor against J-111 antigen was positive with HEp 1 and HEp 2 antigens; and (5) in totals an individual is recorded as positive if positive with any antigen.

type antibody response, as judged by the above criteria. At 1 week 1 serum titered 1:80, 3 were 1:20, 1 was 1:10, and the other was negative. At 4 weeks 1 was 1:160—the highest titer encountered in this study—and none was lower than 1:40. At 7 weeks 1 was 1:80, 2 were 1:40, 1 was 1:20, and 2 were negative.

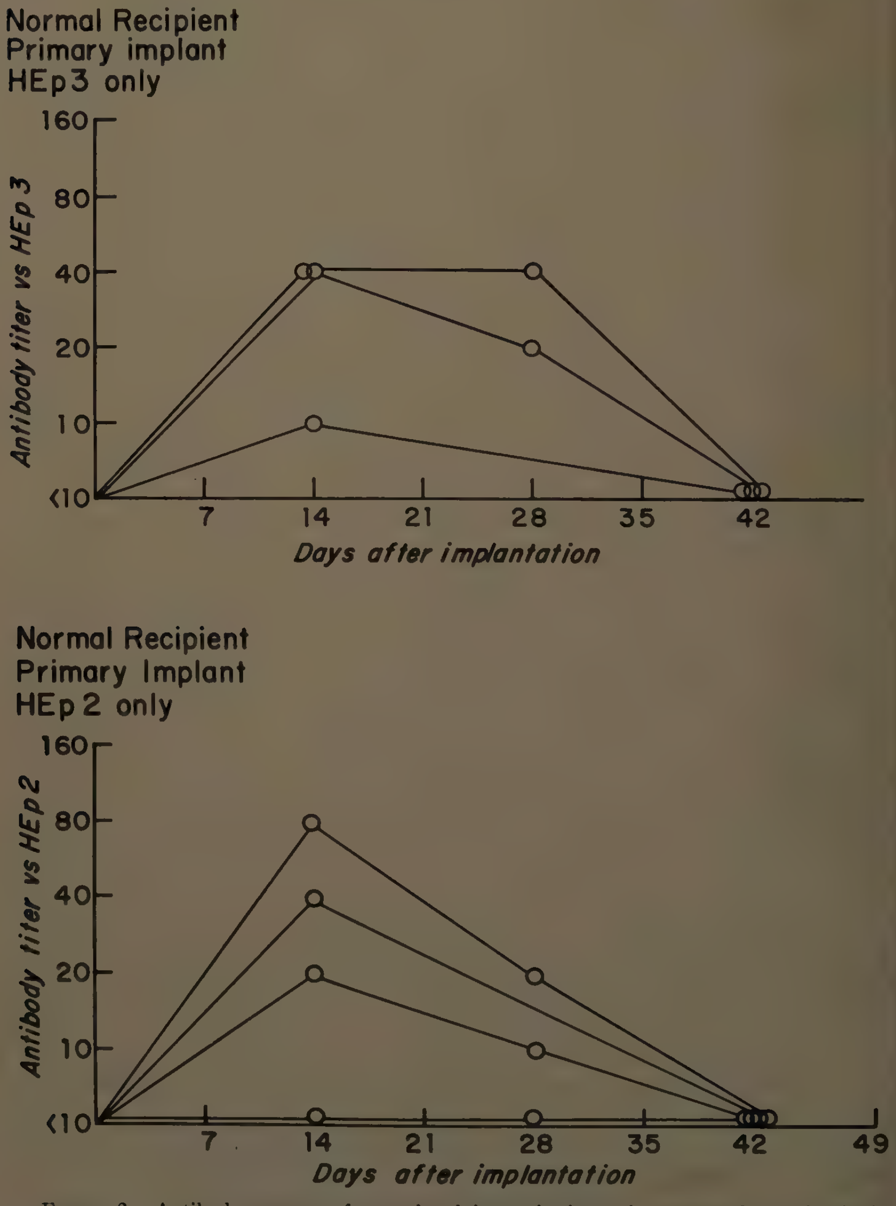
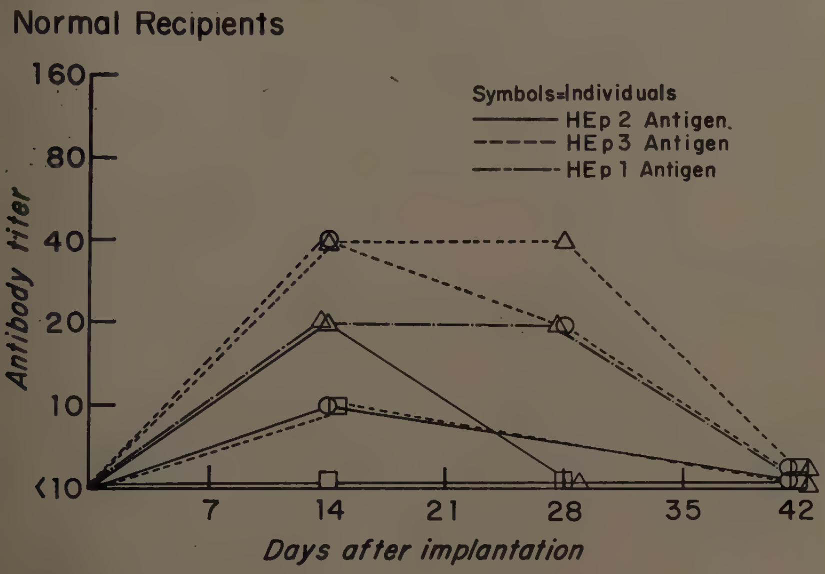


FIGURE 2. Antibody response of normal recipients of primary homotransplants of a single cancer cell line against antigen prepared from the same cell line. Each curve represents an individual recipient.

Although cross reactions between the cancer cell lines that were studied have occurred consistently, there was no cross reaction with the few normal cell lines studied. Two men who received implants of human embryo fibroblasts plus early-passage amnion cells showed no detectable antibody against HEp 2



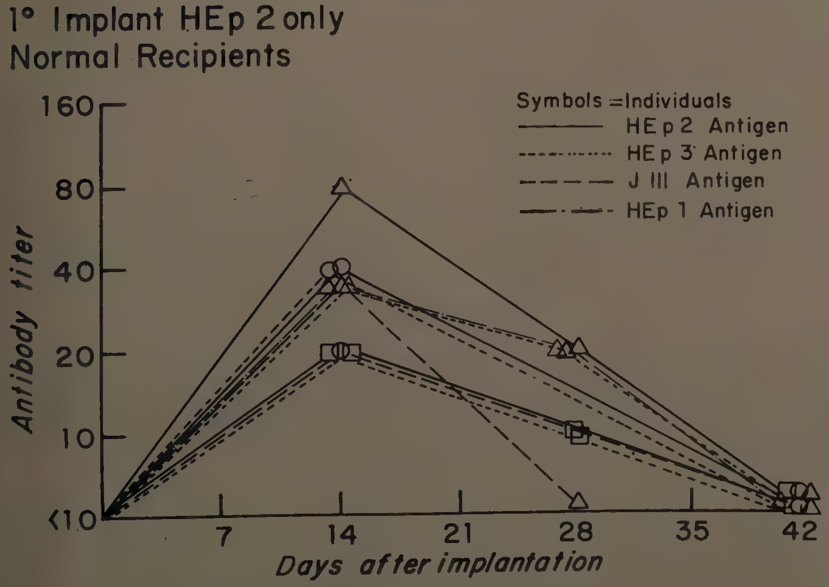


FIGURE 3. Same sera as in FIGURE 2 reacted with antigens from four lines of tissue-cultured cancer cells. Symbols designate individuals. Cross reaction between the four cell lines is striking, but each recipient reacts with the injected cell type as well as or better than with the other cell lines.

or HEp 3 cells (nor against Amnion B cells). Similarly (FIGURE 7), even those sera that showed the greatest response against the cancer cell antigens failed to react with antigens from the presumably normal cells of Amnion B tissue cultures.

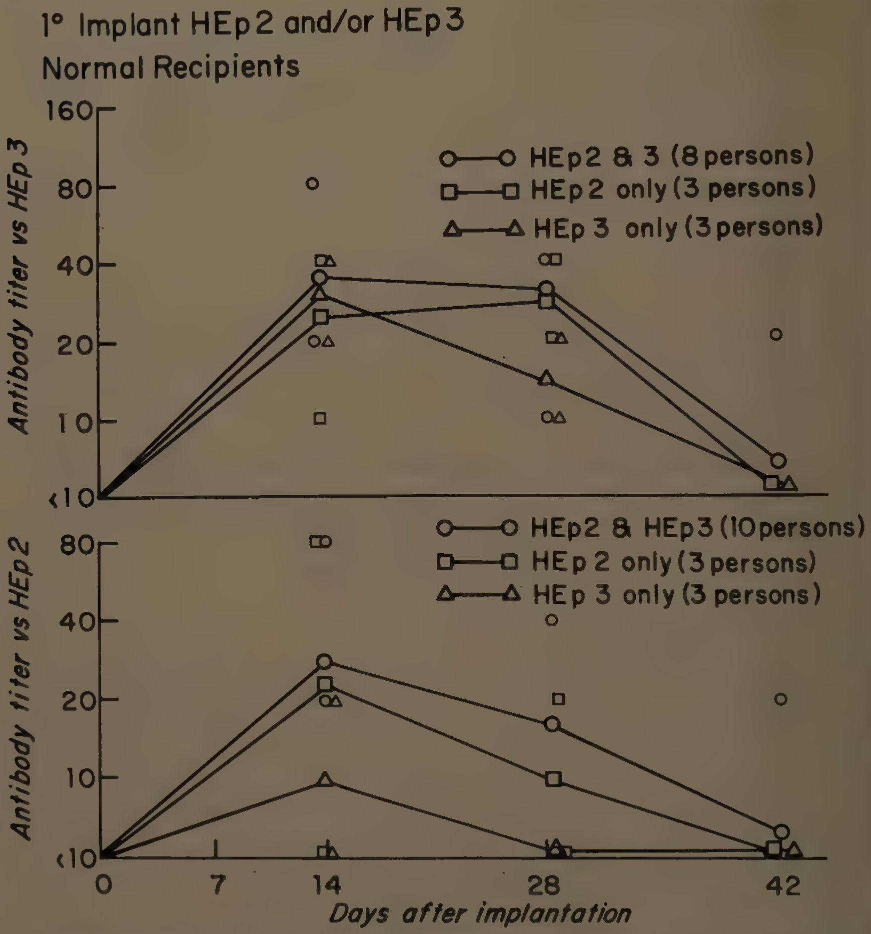


FIGURE 4. Comparison of antibody response of normal recipients inoculated simultaneously with a single cell line (HEp 2 or HEp 3) or with both cell lines. The number of cells implanted was equal for all recipients. All titrations were performed simultaneously. Curves indicate geometric mean titers. Unconnected symbols indicate maximum and minimum titers of individuals in each category. Differences indicate slightly greater response in recipients who received both cell lines.

Antibodies in recipients with advanced cancer. Eleven of 16 cancer patients also showed detectable serum antibodies against 1 or more cancer cell antigens following cancer cell homotransplants (TABLE 2). Among the 5 patients who did not form detectable serum antibody, 3 still had growing homotransplants

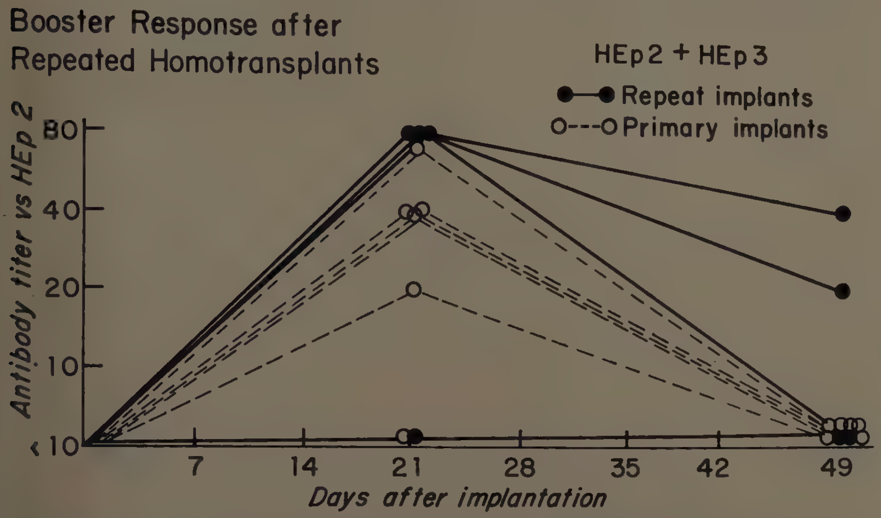


FIGURE 5. Comparison of antibody response of normal recipients after primary and after repeated implants of live HEp 2 and HEp 3 cells. Each curve indicates one recipient. Antibody titer is usually greater and often persists longer after repeat implants, but one recipient of repeat implants produced no detectable antibody.

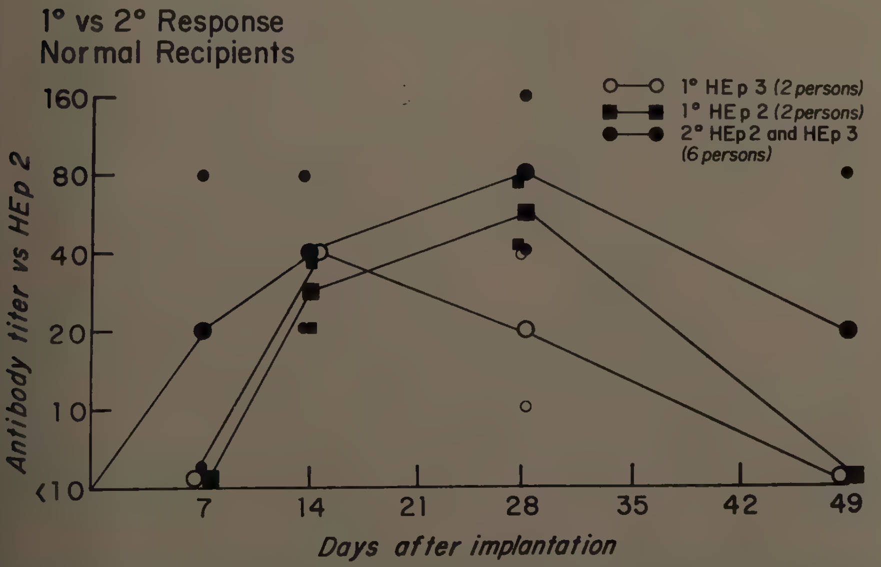


FIGURE 6. Antibody response to homotransplantation of live cancer cell lines in normal recipients who had received killed cancer cell implants four months previously (*black circles*), compared with simultaneous recipients of primary transplants. All titrations were simultaneous. Curves indicate geometric mean titers. Unconnected symbols indicate maximum and minimum individual titers for each category. Antibody at 7 and 49 days indicates a booster type response.

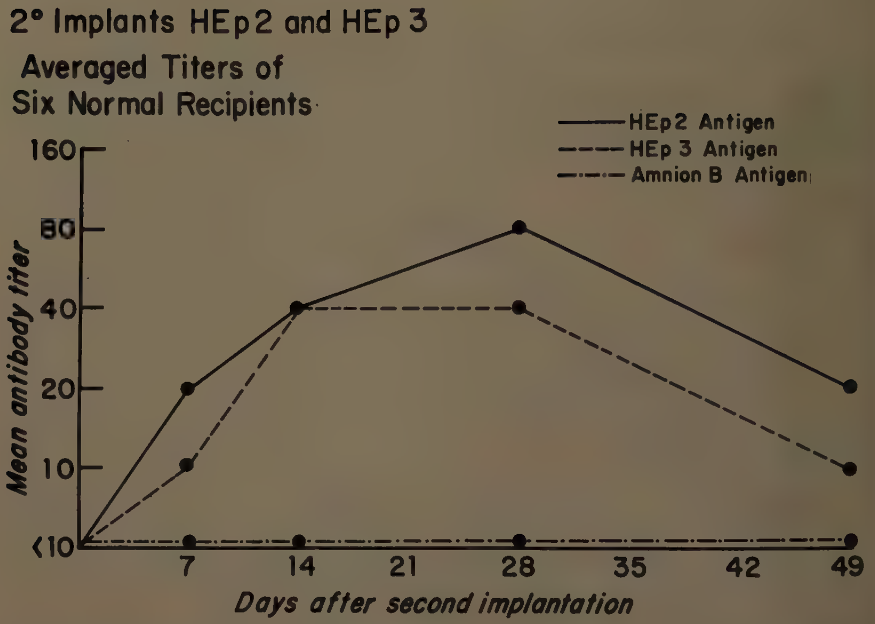


FIGURE 7. Lack of cross antigenicity between Amnion B cells and HEp 2 or HEp 3. Same recipients as in upper curve of FIGURE 6.

TABLE 2

DETECTION OF SERUM ANTIBODY AFTER HOMOTRANSPLANTATION OF HUMAN CANCER CELL LINES IN CANCER PATIENTS

Figures Indicate Number of Individuals

Cell lines implanted (1)	Test antigens	Antibody		
		Yes	No	Remarks
HEp 2	HEp 2 J-111	3 3	3 3	
HEp 3	HEp 2 J-111	1 0	0 1	(2)
HEp 3 and HEp 1	HEp 1 HEp 2 J-111	1 1 1	0 0 0	
HEp 3 and HEp 2	HEp 2 J-111	2 1	1 2	(2)
HEp 2 and J-111	HEp 2 J-111	3 2	1 1	
HEp 2 and HEp 3 and J-111	HEp 2 HEp 3 J-111	1 1 1	0 0 0	
Total: all recipients		11	5	(3)

Remarks: (1) Some patients simultaneously received implants of additional human cell lines of types not studied as test antigens. These include HeLa, F1 amnion, Detroit 6, HAd 1, and HS 1; (2) nonreactor against J-111 antigen was positive with HEp 2 antigen; and (3) in totals an individual is recorded as positive if positive with any antigen.

when the last serum specimen was obtained; therefore, it is possible that absence of circulating antibody might have been due to combination with antigen (in the tumor transplant) rather than failure of antibody production. However, patients who did produce circulating antibody did so (with a single exception) by 2 weeks after implantation in spite of the fact that the implants were growing vigorously at that time. Among the 11 patients who produced serum antibody, only 1 reached a titer of 1:80, but 9 had titers of 1:40. Antibodies were much more persistent in most cancer patients than in normal recipients—possibly because the prolonged growth of the implants provided a continuing antigenic stimulus (FIGURES 8, 9, and 10).

In only one patient has it been possible to study antibody responses following both primary and repeat implants (FIGURE 9). There was an accelerated antibody response after the second implant.

Relationship of serum antibody to transplantation immunity. In the normal recipients there was in general a temporal relationship between antibody response and homotransplant rejection. The transplants produced subcutaneous nodules consisting of cells of the implanted type infiltrated by an overwhelming inflammatory reaction. Most biopsies at 7 days contained the implanted cells, but more than half of biopsies taken at 14 days showed only the rejection reaction. Regression was almost always complete by 28 days.[7] Serum antibody level reached its peak at the height of the rejection response, persisted until the implant was gone, and disappeared shortly thereafter. The booster type response of serum antibody after repeat implants also is consistent with the accelerated rejection of such implants. However, a serum antibody response is not a requisite for implant rejection, for in the 2 individuals who failed to produce detectable serum antibodies after primary implants there was no difference in size or time of rejection of the implants as compared to the 5 simultaneously implanted recipients who did develop serum antibodies. Similarly, when the individual who produced no circulating antibodies even after repeat implants was compared with the simultaneously implanted recipients who did produce circulating antibodies, there was no difference in the rapidity of implant rejection.

In the cancer patient recipients there was no consistent relationship between serum antibody and rejection of the cancer cell homografts. This is illustrated in FIGURES 8 to 11, which compare size of the subcutaneous nodules of homotransplanted cancer cells with serum antibody levels in individual patients. In some patients there was a temporal relationship, with antibody appearing as the implant reached its maximum size and disappearing shortly after rejection was completed (FIGURES 8 and 9). However, in other patients, antibody appeared at the expected time, but disappeared as the homotransplant continued to grow (FIGURE 10), or continued to circulate concurrently with continued growth of the transplanted cancer cells (FIGURE 11). This last situation was demonstrated most strikingly by the continuous presence of serum antibody at levels of 1:20 to 1:80 in the only patient in whom the homotransplanted cancer cells have been observed to metastasize.[8] In these cancer patients primary implants produced no inflammatory response, and the tardy rejection reaction was characterized by round cell infiltration. Thus there was no consistent correlation between serum antibody levels and the rejection response as a whole, or the host cell reactions that accompanied it. It cannot be inferred that serum antibody plays no part in the rejection reaction, but it seems evident that serum antibody is not the predominant mechanism.

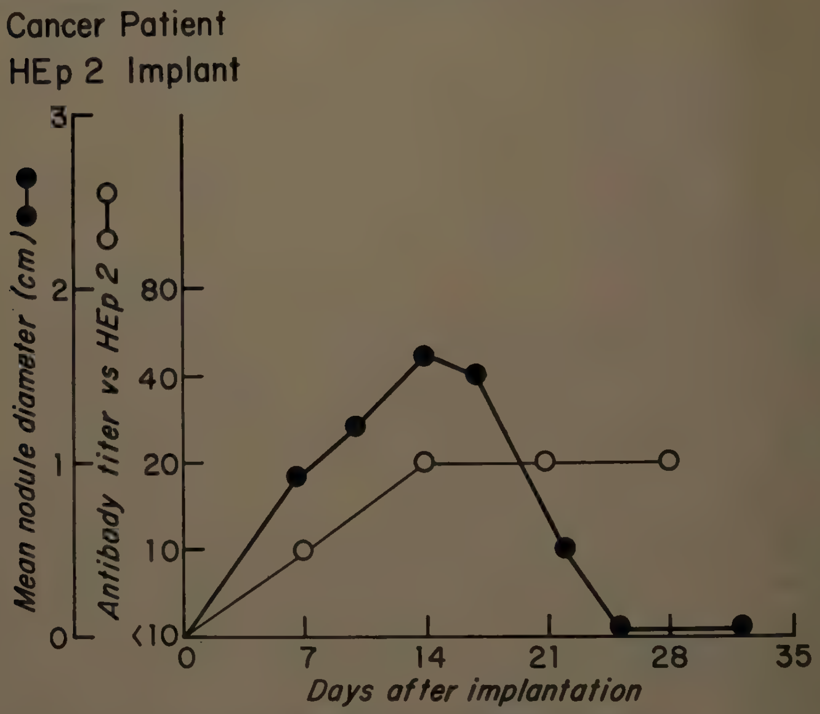


FIGURE 8. Comparison of antibody response and homotransplant growth in cancer patient 205732. Apperance of antibody coincides with rejection of implant.

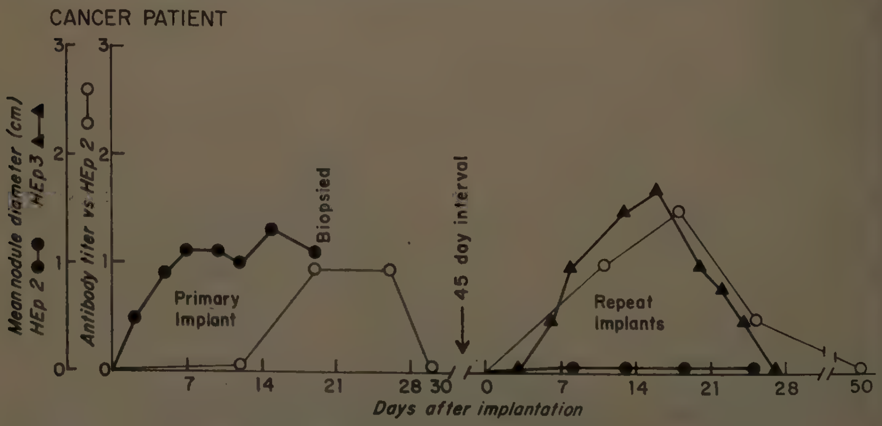


FIGURE 9. Comparison of antibody response and homotransplant growth in cancer patient 58-3082 after primary and repeat transplants. Appearance of antibody coincides with regression of implants both times. Note earlier appearance of antibody after second implant.

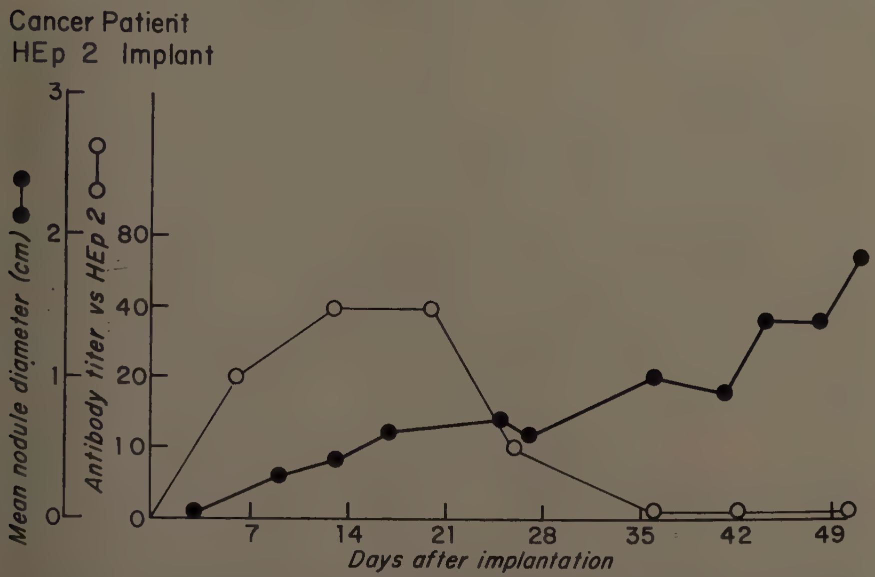


FIGURE 10. Comparison of antibody response and homotransplant growth in cancer patient 126790. Appearance of antibody as in FIGURES 8 and 9 but transplant continued to grow (confirmed by punch biopsies on days 28 and 66) and antibody disappeared.

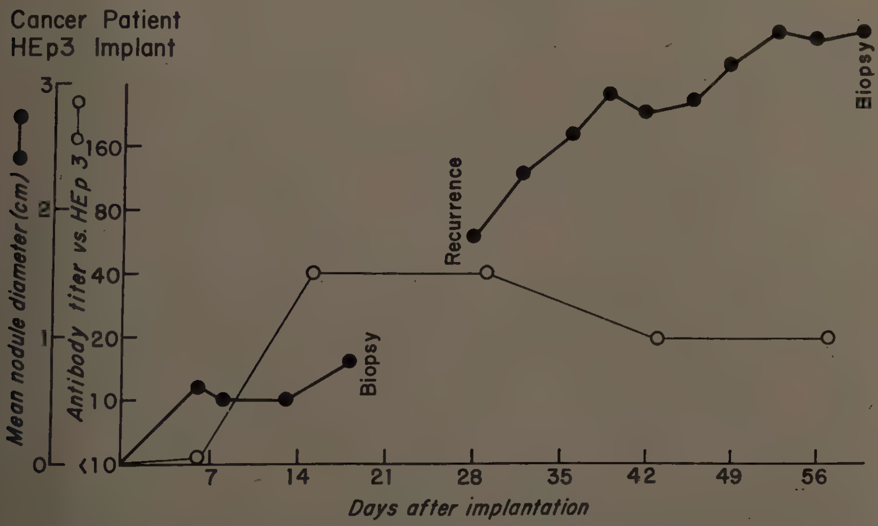


FIGURE 11. Comparison of antibody response and homotransplant growth in cancer patient 148103. Both biopsies showed solid mass of healthy HEp 3 cells with no host cell reaction. Transplant grew progressively for two months in spite of circulating antibody.

Discussion

It seems probable that this demonstration of circulating antibody by the tanned-erythrocyte technique after failure by previous methods merely indicates a greater sensitivity of this technique rather than an unusual character of the antibody. The high sensitivity of this technique is well documented and is illustrated in the present study by the fact that heterologous systems (rabbit antihuman cancer cell sera) show antibody titers in the range of 1:20,000 by this method.

The test antigen for these studies is crude and completely uncharacterized as to chemical or physical nature. Nor is it known what cellular components are present. It was found, however, that the discarded sediment from the antigen preparations was not antigenically active. Comparisons with the ergastoplasmic antigen of Toolan[10] and the cytolipin of Rapport and Graf[6] are of obvious interest, but have not yet been attempted.

The demonstration of circulating antibody to homologous cells in this study does not diminish the plausibility of the thesis[3] that the homotransplant rejection reaction resembles the delayed hypersensitivity type of response rather than immune states characterized by circulating antibodies. The apparent unimportance of circulating antibody in the rejection mechanism is suggested above; even in tuberculosis, the classic example of the delayed hypersensitivity response, circulating antibody can be demonstrated by the tanned-cell hemagglutination technique.[2] These *in vitro* studies strengthen the impression, gained from study of "second-set" reactions,[7] that all the cancer cell lines studied to date have an antigenic overlap, which does not extend to normal amnion cells. The data, however, do not permit an opinion as to whether or not this common antigenicity among the cancer cell lines is related to the neoplastic nature of the cell or is coincidental.

The participation of blood-type antigens in these reactions was considered, but seems adequately excluded by several lines of evidence. The HEp 2 and HEp 3 cell lines both came from type O Rh-positive patients, HEp 1 was from a group A Rh-positive patient, and J-111 from a group A Rh-negative patient, yet there was cross antigenicity between these 4 cell lines (the amnion tissue cultures originated from pools of placentas, and individual sources could not be traced). Comparison of type-A versus type-O recipients and Rh-positive versus Rh-negative recipients revealed no difference in height or duration of antibody response. The adsorption of antibody-positive human sera with washed pooled human types A and B erythrocytes did not alter their antibody titer for either HEp 2 or J-111 antigens. Preliminary studies by the mixed-agglutination and fluorescein-labelled antibody techniques have given no indication that the A blood-type antigen persists in established cell lines from type-A patients.

Summary

Antibody was demonstrated in sera of recipients of human cancer cell homotransplant by the tanned-erythrocyte agglutination technique using as antigen a crude saline extract of tissue-cultured cancer cells (HEp 1, HEp 2, HEp 3, J-111).

Almost all of the normal recipients had detectable antibody at serum dilution titers of 1:10 to 1:80 at 2 and 4 weeks after primary implantation, but had none (<1:10) by 6 or 7 weeks. After repeat implants, antibody sometimes appeared by 1 week and usually remained positive at 6 to 7 weeks. Inoculation of killed cancer cells rarely caused a detectable serum antibody response, but subsequent implantation of live cancer cells gave a booster-type response.

There was a high degree of cross antigenicity among the 4 cancer cell lines studied, but no evidence of cross antigenicity between the cancer cell lines and tissue-cultured normal human amnion cells.

Among recipients who were ill with advanced cancer, a majority had a similar antibody response. None had antibody prior to homotransplantation.

The presence of titers of serum antibodies could not be related to transplant rejection, since the two normal recipients who failed to produce antibody rejected just as promptly as other normal recipients, and in some cancer patients the homotransplants continued to grow for several weeks in spite of the presence of circulating antibody.

Acknowledgments

We are greatly indebted to Virginia Babcock for maintenance of tissue cultures used for antigen preparation and to the normal and patient volunteers at the Ohio State Penitentiary, Columbus, Ohio, and at Memorial Center for Cancer and Allied Diseases, New York, N. Y., respectively, who served as homograft recipients.

References

1. BOYDEN, S. V. 1951. The adsorption of proteins on erythrocytes treated with tannic acid and subsequent hemagglutination by anti-protein sera. J. Exptl. Med. **93:** 107–120.
2. COLE, L. R. & C. B. FAVOUR. 1955. Correlations between plasma protein fractions, antibody titers, and the passive transfer of delayed and immediate cutaneous reactivity to tuberculin PPD and tuberculopolysaccharides. J. Exptl. Med. **101:** 391–420.
3. LAWRENCE, H. S. 1947. Similarities between homograft rejection and tuberculin-type allergy: a review of recent experimental findings. Ann. N. Y. Acad. Sci. **64**(5): 826–835.
4. OSGOOD, E. E. & J. H. BROOKE. 1955. Continuous tissue culture of leukocytes from human leukemic bloods by application of "gradient" principles. J. Hematol. **10:** 1010–1022.
5. MOORE, A. E., L. SABACHEWSKY & H. W. TOOLAN. 1955. Culture characteristics of four permanent lines of human cancer cells. Cancer Research. **15:** 598–602.
6. RAPPORT, M. M., L. GRAF, V. P. SKIPSKI & N. F. ALONZO. 1959. Immunochemical studies of organ and tumor lipids. Cancer. **12:** 438–445.
7. SOUTHAM, C. M. & A. E. MOORE. 1958. Induced immunity to cancer cell homografts in man. Ann. N. Y. Acad. Sci. **73**(3): 635–651.
8. SOUTHAM, C. M., A. E. MOORE & C. P. RHOADS. 1957. Homotransplantation of human cell lines. Science. **125:** 158–160.
9. STAVITSKY, A. B. 1954. Micromethods for the study of proteins and antibodies. I. Procedure and general applications of hemagglutination and hemagglutination-inhibition reactions with tannic acid and protein-treated red blood cells. J. Immunol. **72:** 360–367.
10. TOOLAN, H. W. & R. A. WALLACE. 1958. The relative ability of cell-free fractions of the transplantable human tumor, HEp #3, to produce HEp #3 cytotoxins in rats. Cancer Research. **18:** 698–705.
11. WADSWORTH, A. B. 1947. Standard Methods of the Division of Laboratories & Research of the New York State Department of Health, 3rd ed. : 226. Williams & Wilkins. Baltimore, Md.

PREPARATION OF A TRANSPLANTATION ANTIGEN FROM EPIDERMAL CELLS*

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INTRODUCTION

For a long time we have been interested in the use of epidermal cells in the study of problems of grafting. Starting from previous experiments of Billingham and Sparrow (1955), we studied the effects of intravenous injections of epidermal cells in adult animals on the survival time of skin homografts from the same donors.

In the adult rabbit as well as in the mouse, these preliminary injections, made a few days before the graft, produced an important prolongation of the survival time of these skin homografts. Our experiments on the rabbit thus confirmed the results of Billingham and Sparrow.

Unfortunately, these experiments with basal epidermal cells, isolated with trypsin and washed several times, were characterized by a high mortality.

We first had a rather high mortality at the time of, or shortly after, intravenous injections. This mortality could be reduced by additional washings of the cells, but the effectiveness of the cells on the survival of the homografts simultaneously decreased. However, when rabbits were used, there also was a delayed mortality that reached an average of 30 per cent.

These experiments emphasized an additional risk in all our attempts at inducing homograft tolerance with living cells (namely, runt disease and homologous disease).

As have many others, we tried to prepare cell-free extracts capable of eliciting, if possible, a total tolerance, or at least a more or less prolonged survival time, of the skin homografts.

With this objective in mind, we have investigated the problem in two ways:

In one field of work we use extracts of gastric and duodenal mucosa. These extracts, if not identical, are at least very close to the blood group substances or prepared by the same technique. When injected intravenously, they effect a very significant survival of skin homografts from the donor of the extracts. Like the blood group substances, these extracts have the property of inhibiting hemagglutination. They are heat-stable and do not produce transplantation immunity. This work is still going on and has been reported partially elsewhere (Albert and Lejeune-Ledant, 1958, 1959).

The other field of this work consisted of an attempt to prepare extracts containing antigens capable of producing an immunological response in adult animals and inducing tolerance in immature animals. For this we chose epidermal cells. The first part of this work is the subject of our present communication.

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All previous attempts at extracting transplantation antigens have involved spleen, lymph nodes, or thymus cells. Billingham *et al.* (1958) published the pioneer work, using an elegant technique for the preparation of cell-free extracts. Since then various methods have been used; among others, we note those of Haskova and Majer (1959) and of Oth and Castermans (1959). We always have been tempted to utilize epidermal cells, even when injecting living cells, because of their powerful antigenic activity.

It is known from the work of Billingham *et al.* (1959) that the transplantation immunity reaction to a skin homograft is proportional to the size of the graft. Nevertheless, a micrograft composed of about a thousand epidermal cells (5×10^4) gives a transplantation immunity that will last for a long time.

Despite the preliminary apparent difficulties, the assay appeared sufficiently interesting for a continuation of our investigations. The course followed was: (1) preparation of epidermal cell extracts capable of producing transplantation immunity reaction; (2) determination of the type and importance of the response to injections of the substances, especially in relation to the dosage, the route and, further, repetition of these injections; (3) attainment of a more or less high tolerance by injections at birth; and finally, (4) chemical analysis of the complex extracts, with a view to identifying the active antigen substance or substances.

In this communication, we refer only to the first three steps.

MATERIAL AND METHODS

Selection of Animals

In the study of antigenic transplantation properties we used adult animals; in the study of tolerance we used newborn mice injected at birth.

Mice. Swiss albinos and C57 Black were used in combination, either one as donor or recipient. The Champagne strain was used only as donor to the Swiss albino. The three strains, Swiss albino, C57 Black, and Champagne, were all inbred for several years at our laboratory.

The results of the median survival times of skin homografts exchanged between them are almost equal:

Donor	Recipient	
Swiss albinos	C57 Black	10.2 days (±0.4)
C57 Black	Swiss albinos	10.5 days (±0.6)
Champagne	Swiss albinos	9.5 days (±0.4)

Series of 25 mice were used for each group of experiments.

Rabbits. "Giant of Flanders" rabbits were chosen so as to realize a real genetic disparity. The donors were brownish-grey; the receptors, albino. Weight varied between 2.5 and 3.5 kg. Every experiment was performed also with controls of both species.

Preparation of Extracts

The preparation technique may be schematized as two main steps, the preparation of basal epidermal cells and the extraction of the transplantation antigen from these cells.

Preparation of basal epidermal cells. Thin fragments of skin are taken from the ears of the rabbits (FIGURE 1). Hemostasis is effected by means of temporary compression. The wound, powdered with sulfamides, is left without any dressing; healing is rapid and is complete within twelve days.

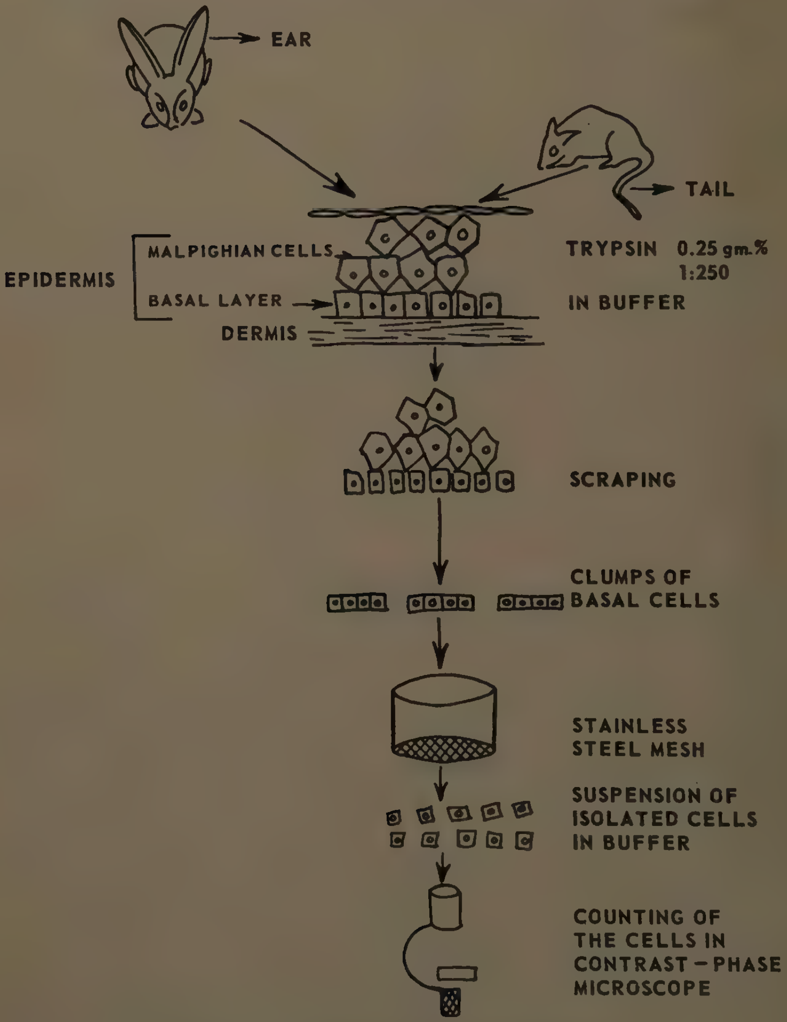


FIGURE 1. Preparation of basal epidermal cells.

The skin is removed from the tail of the mouse. After a circular incision around the base and an incision along the tail have been made, a trapezoidal piece of skin may be removed easily.

The fragments of skin are immersed immediately in a balanced solution at *p*H 6.8. This solution has the following composition: 0.137 *M* NaCl, 0.0026

M KCl, 0.0081 *M* Na_2HPO_4, 0.00147 *M* KH_2PO_4, 0.0009 *M* $CaCl_2$, and 0.00048 *M* $MgCl_2 \cdot 6H_2O$.

The presence of ions Ca and Mg protects the antigens against the action of trypsin. The trypsin is added to obtain a final concentration of 0.25 gm./100 ml. The incubation is performed at 37° C. for 30 to 35 min.

The epidermal sheet then is removed easily from the dermis and placed on a microscope slide so that the basal layer may be scraped gently and collected in a solution of the same composition but without trypsin. The cells are passed through a finely meshed stainless steel sieve. They are then pipetted vigorously several times, to obtain a suspension of most perfectly isolated cells. They are counted 6 times with a hemocytometer by phase-contrast microscopy and the average is calculated.

Extraction of the transplantation antigen. After various attempts we have adopted the following method (FIGURE 2).

The cellular suspension at 37° C. is submitted again to the action of trypsin at the same concentration and in the same buffered solution as described above. The treatment lasts for 5 to 20 min. and is stopped at the time of cellular destruction.

This stage of preparation requires a regular microscopic follow-up. The incubation is cut off at the time that approximately only one fifth of the initial amount of cells is perceivable. Even then, cytoplasm and nuclei have fuzzy contours. Moreover, many cell-free particles may be observed.

All manipulations now must be performed at +4° C.

The next step is a 10-min. centrifugation at 1500 g. The supernatant liquid is discarded and the precipitate collected.

To eliminate the trypsin completely the precipitate is washed in a 0.15 *M* NaCl solution and again centrifuged at 1500 g. This procedure is repeated 3 times. The antigenic sediment now is dissolved in an aqueous solution of 1 vol. 0.15 *M* NaCl and 2 vol. of water and centrifuged at 1500 g.

The antigenic substances are suspended in pure water and centrifuged for 10 min. at 2500 g. A viscous sediment is thus obtained that may be cut with scissors.

This sediment is homogenized at high speed (20,000 rpm) for 15 sec. After homogenization the preparation is centrifuged at 1500 g for 10 min.

The supernatant liquid is collected, buffered, and centrifuged at 25,000 g for 1 hour at +4° C.

The final precipitate is suspended in a buffered solution of 0.15 *M* NaCl at *p*H 6.2 to 6.3.

The volume of this suspension is 0.5 cc. for the adult mice and 10 cc. for the rabbits.

That the concentration is directly proportional to the number of the cells initially present explains the importance of the precision in cellular counting at the beginning of the preparation. It may be seen that, after the stage of cell destruction with trypsin, our technique, with slight variations, is similar to that used by Billingham *et al.* (1958) in the preparation of their antigenic matter from spleen, lymph nodes, and thymus cells.

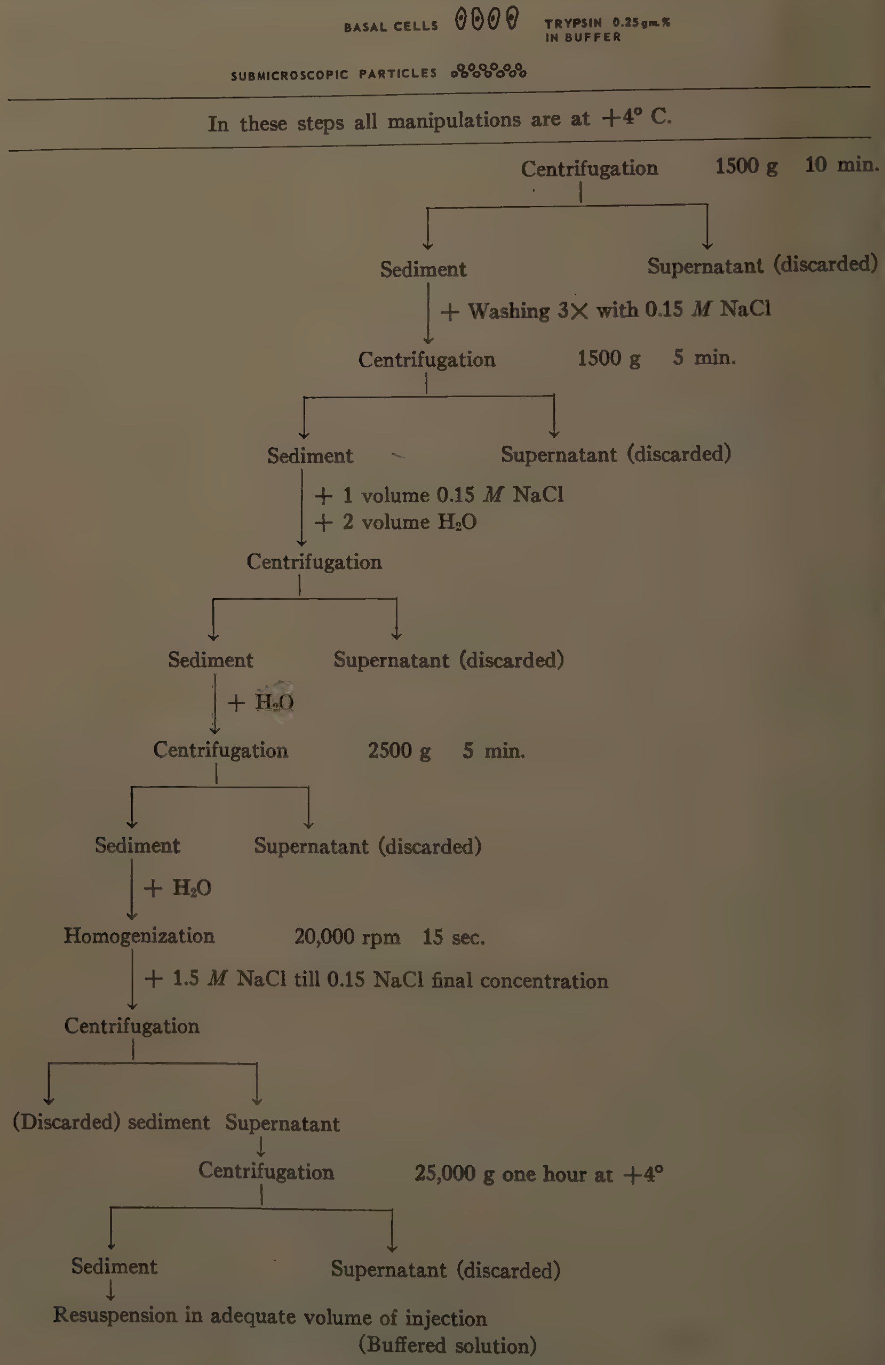

BASAL CELLS
TRYPSIN 0.25 gm.% IN BUFFER
SUBMICROSCOPIC PARTICLES
In these steps all manipulations are at +4° C.
Centrifugation 1500 g 10 min.
Sediment
Supernatant (discarded)
+ Washing 3× with 0.15 M NaCl
Centrifugation 1500 g 5 min.
Sediment
Supernatant (discarded)
+ 1 volume 0.15 M NaCl
+ 2 volume H2O
Centrifugation
Sediment
Supernatant (discarded)
+ H2O
Centrifugation 2500 g 5 min.
Sediment
Supernatant (discarded)
+ H2O
Homogenization 20,000 rpm 15 sec.
+ 1.5 M NaCl till 0.15 NaCl final concentration
Centrifugation
(Discarded) sediment
Supernatant
Centrifugation 25,000 g one hour at +4°
Sediment
Supernatant (discarded)
Resuspension in adequate volume of injection (Buffered solution)

FIGURE 2. Extraction of the T-epidermal antigen.

Antigenic Transplantation Activity Test

The following test has been proposed by Billingham *et al.* (1956).

A single intraperitoneal injection of the substances suspected of antigenic activity is made. Three or four days later the recipient is grafted with donor skin. At the end of 6 days the graft is inspected and removed for histological examination. For the most accurate histological diagnosis, the homograft is cut into 4 equal pieces, and at least 30 sections are systematically examined. If there is any doubt, supplementary sections are made.

The histological aspects are interpreted by following the criteria of Billingham *et al.* (1954), and are noted as 100 per cent survival of the epidermis, 50 per cent survival of the epidermis, or 0 per cent survival (total breakdown).

The degree of round cells as well as of hemorrhagic infiltration is noted.

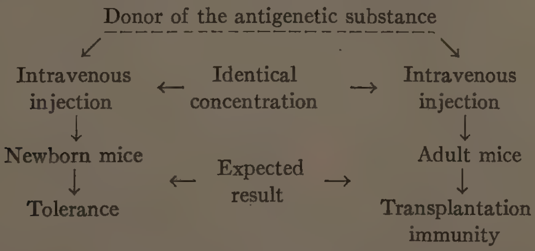
Various routes of injection, intraperitoneal, intravenous, and intradermal, are used for comparative study.

Skin Homografts

All skin homografts on mice as well as on rabbits have been performed by the well-known method of Billingham and Medawar (1951).

Newborn Swiss albino mice are injected intravenously (technique of Billingham and Brent, 1957) in the first hours after birth, the antigenic component being obtained from either C57 Black or Champagne mice. The same concentration of antigenic material is injected intravenously into adult controls.

The schema of the experiment may be given as follows:



At the present time, the control injection into the adult animal is the only possible means of verifying the antigenic activity of the extract used.

Another complementary experiment has been made, again by injection into newborn mice, but this time of living epidermal cells. All the baby mice injected with either extracts or living cells are challenged 6 weeks later with skin homografts of the same donors.

RESULTS

For a comparison of results, the degree of antigenic activity is always estimated as a function of the number of epidermal cells used for the preparation of our extracts.

Adult Animals

Mice. With the combination of Swiss albinos and C57 Black, one as donor and one as recipient, we observed in all cases an actual sensitization of the recipient characterized by an accelerated breakdown of the skin homograft on the sixth day.

The percentage of survival of the graft epidermis, as indicated in TABLE 1, decreased as a function of the concentration of the injected extracts. Thus an extract containing 10^7 cells provoked a large second set in 13 cases and a much more violent one in 7 others, from a total of 25 cases.

TABLE 1
BIOPSY ON THE SIXTH DAY

Intraperitoneal; epidermal cells, 10^6	No. animals	Survival of epidermis (%)	No. animals	Survival of epidermis (%)	No. failures
1	5	75	18	25	2
2*	10	50	11	25	4
4	9	50	12	25	4
5	14	50	10	0	1
7.5*	13	25	8	0	4
10	13	25	7	0	5

* Adult mice (8 weeks): C57 Black → Swiss albinos, Swiss albinos → C57. Chemical lysis of the cells: trypsin.

TABLE 2
RABBITS*

Donor No.	Recipient No.	Dosage ($\times 10^6$)	*p*H	Volume (cc.)	Macroscopic disintegration	Day 6 survival of epidermis (%)	Recipient weight (kg.)
L 313	D 191	0.6	6.5	2.5		25	2.5
L 295	D 195	1.5	6.5	6	Visible on day 7	25	2.8
L 316	D 194	4.7	6.5	3.5		0	3.2
L 296	D 196	5	6.4	8		100	2.9
L 325	D 204	5	6.2	10	Visible on day 7	0	2.8
L 320	D 167	15	6.3	10	Visible on day 7	0	3.1
L 295	D 200	15	6.3	9.5	Visible on day 6	0	3.5
L 326	D 205	10	6.2	10		50	3.3
L 327	D 206	10	6.3	9.8		0	2.8
L 328	D 209	3	6.2	10		25	3.4
L 329	D 210	10	6.2	9.4		25	3.5
L 322	D 214	12	6.2	9.7		0	2.7
L 333	D 215	7	6.2	9.9	Visible on day 6	0	2.5
L 334	D 227	15	6.2	9.5		100	3.2

* Intravenous injection of a single dose.

We had five negative results. The interpretation of these negative results, observed also by other workers, when a fault of technique may be excluded, is rather difficult at the present stage of our progress in experimentation.

Rabbits. An intravenous injection of 6×10^5 cells into the rabbit resulted in a survival of 25 per cent of the epidermis of the skin homograft to the end of the sixth day. At the same time, a marked infiltration of round cells was observed at the base of the graft. When stronger concentrations were injected (TABLE 2), such as 15×10^6 cells, the reaction became macroscopically visible.

At the end of the sixth day, hemorrhagic infiltrations were noted, especially in the case L 295 → D 200 and L 333 → D 215. Thus a typical second-set reaction was observed.

Two failures have been detected. In these cases the epidermis of the graft at the end of the sixth day was in hyperplastic phase, just as in the control animals.

We emphasize that failures have been noticed: one occurred with an average dose of 5×10^6 cells (L 296 → D 196), the other with a very high dose of 15×10^6 cells (L 334 → D 227).

New series of experiments attempt to discover the reason for these abnormal results. It ought not to be forgotten, as other experiments confirm, that rabbits show poor homogenous response to various antigenic stimuli, as far as immunological studies are concerned.

Tolerance Induction Tests

It is still too early to arrive at a definite conclusion concerning tolerance, but we have never observed a perfect tolerance in Swiss albino mice injected at birth with a dose of 10^6 C57 Black cells. Nevertheless, we observed some prolonged survivals of skin homografts, but their number and durations are not statistically significant. Additional experiments now are being undertaken with higher concentrations and repeated injections.

The adult control animals in all the cases showed a definite transplantation activity of the extracts.

It is perhaps worthwhile to point out that, of all the mice injected at birth with epidermal cells, none manifested the slightest tolerance. All homografts disintegrated in 10 days.

COMMENTS

Antigenic Activity of the Cell-Free Extracts

We did not arrive by a direct route at the definitive method of preparing extracts. The first question to answer was: Is it or is it not possible to use epidermal cells for the preparation of cell-free extracts having a sufficient antigenic activity to produce a transplantation immunity phenomenon?

To settle this point, the most simple technique was used: the basal epidermal cells, isolated after incubation with trypsin, were ground with fine glass powder in a sterile mortar in normal saline at +4° C. After centrifugation at moderate speed (1500 g) for 5 min. a sediment was formed of the very fine glass powder with some adherent organic particles. The opalescent supernatant, in which no living cells could be detected, was used for the antigenicity test. A single intraperitoneal injection of the extract was followed after 3 or 4 days by a skin homograft from the same donor.

Fifty Swiss albino mice were used as recipients for these experiments. They had an intraperitoneal injection of extracts at a concentration of 5×10^6 cells coming from C57 Black. Six of these mice died within 24 hours, 17 others during the 3 days after the injection (TABLE 3). This was a prohibitive mortality rate. All the survivors showed an accelerated disintegration of their homografts on the sixth day.

We could be sure that cell-free extracts of epidermal cells, even when obtained by this very simple technique, are able to induce a typical response of transplantation immunity. However, a great part of the active antigenic substances cannot be recovered; furthermore, the toxicity of this kind of extract precluded all practical utilization.

The next step was to produce a more efficient purification of our antigenic extracts.

A second type of preparation was inspired by the technique used by Billingham *et al.* (1958) for their extracts of spleen, lymph nodes, and thymus cells.

The basal epidermal cells isolated by treatment with trypsin and washed several times in normal saline were destroyed mechanically by homogenization at 20,000 rpm for 15 sec. All the further manipulations were identical with those described above and completed our last and probably definitive technique.

TABLE 3

No. animals	Survival of epidermis of graft (%)
11	50
16	0

TABLE 4

Cellular destruction	No. animals	Survival of epidermis (%)	No. animals	Survival of epidermis (%)
Mechanical action	6	50	19	0
Chemical action	2	50	23	0

The only difference between the two last techniques is that, in the first, the cells are destroyed mechanically and in the second, by the action of trypsin.

At first we had the impression that the extracts prepared by one or the other of our techniques, which produce nearly identical immunological responses (TABLE 4), were also chemically identical. However, the trypsin used in the second technique for the destruction of the epidermal cells eliminates a certain proportion of proteic substances. This may be verified by comparing the amount of nitrogen in the two extracts. The amount actually is lower in the extracts prepared with trypsin. This fact should make the approach to the chemical study of the antigens easier. In so far as the antigenic activities of the two extracts are concerned, if there should be any difference between them, the extract prepared with trypsin would be the more effective.

Using the combination of C57 Black and Swiss albino mice in 25 cases, we observed a poor survival of the epidermis of the graft after a dose of 5×10^6 cells. For this reason we have definitively adopted tryptic action as a means of cell destruction. The results obtained with this technique are indicated in TABLES 1 and 2.

The smallest active doses are of the orders of 10^6 basal cells for mice and of 6×10^5 cells for rabbits.

Biopsy has shown in every case a poor survival of the homograft epidermis on the sixth day. At the same time the base of the graft reveals a distinct lymphocyte infiltration. This is in marked contrast with the control animals homografted without any pretreatment. In these cases the epidermis is hyperplastic at the sixth day.

If, on the other hand, the concentration of the injected extract is increased, the antigenic reaction is more violent. The epidermis of the graft is destroyed completely and the base of the graft shows an important hemorrhagic infiltration.

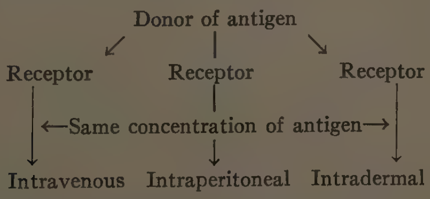
In rabbits as well as in mice in several cases macroscopic signs of disintegration at the sixth day were noted. In rabbits this is characterized by an aspect of a "white graft" from a dose of 15×10^6 cells and, in mice, by a large hemorrhagic infiltration from a dose of 10^7 cells.

Toxicity

The injections of extracts obtained by the first technique showed a high degree of toxicity. On the other hand, those prepared by mechanical or chemical destruction of the cells have never produced the slightest mortality. A number of mice injected with a very high concentration of extracts (35×10^6 cells) was watched for a long time for delayed alteration of general health with late mortality. All the animals are still in excellent state after more than 3 months. These observations contrast strongly with those reported elsewhere, when we were using living epidermal cells. At that time, during or immediately after the intravenous injection the mortality reached about 30 per cent. In our prolonged homograft survivals after injection of these cells we observed a delayed mortality with progressive alteration of the general health in 30 per cent of the survivors.

Efficiency of the Route of Injection

The importance of the route of administration has been tested in 3 different ways: intraperitoneally, intravenously, and intradermally. Three groups of 10 each of Swiss albino mice were injected by 3 different routes with an extract of 10^6 epidermal cells from C57 Black mice:



Injections of our extracts at the same concentration by the three routes produce typical immunological responses. However, as shown in TABLE 5, the intradermal injection in the region corresponding to the future bed of the homograft induces a much more violent breakdown of the graft.

This experiment has been repeated on rabbits with the same result.

Inactivation

The extracts are not heat-stable. In all types of experiments used, heating at 56° C. for 30 min. abolishes entirely the antigenic activity of transplantation immunity.

Induction of Tolerance

Intravenous injections of our extracts at various concentrations (from 10^6 to 5×10^6 cells) into newborn mice have not induced a complete tolerance. Some mice showed a certain prolongation of the survival of their homografts, but thus far the number and the importance of the survival times are not important enough to be statistically significant.

Another demonstration of the relative induced tolerance in newborn mice challenged with a second homograft of the same donor is given by the absence of a second-set reaction at the sixth day.

Injections of higher concentrations of extracts and multiple injections will be attempted very soon.

TABLE 5

	Injection (concentration: 10^6 cells)		
	Intravenous	Intraperitoneal	Intradermal
Day 6 survival of epidermis in 10 mice (%)	50	50	0

The responses to such experiments are very important. We urgently need to know whether the antigenic substances of cell-free extracts are able to induce tolerance.

CONCLUSIONS

Cell-free extracts from the basal layer of the epidermis obtained by different techniques produce in adult rabbits as well as in mice a strong transplantation-immunity response.

The antigenic component obtained by this means is very active. A dose of extract of 10^6 cells gives, in both species, a typical second-set reaction by the sixth day.

The extracts are efficient when administered by all the usual routes of administration: intraperitoneal, intravenous, and intradermal. However, intradermal injection of the extract at the site of the future bed of a skin homograft causes a much more violent breakdown.

The preparation of these extracts by simple grinding gives a product of very high toxicity but with an actual antigenic activity. The preparation by mechanical or chemical destruction of cells produces a component of apparently the same antigenic activity. The cellular destruction by the action of trypsin has, however, the advantage of eliminating a great part of the proteins, thus giving an antigenic component perhaps more favorable to further chemical investigation.

In so far as the antigenic activities of both these extracts are concerned, if there is any difference between them it should favor the extract prepared by the action of trypsin.

The intravenous injection of our extracts into mice during the first hours after birth thus far has not induced any sign of total tolerance of skin homografts made during the adult life of these animals. In some cases, however, such injection produced a prolonged survival of the skin homograft, but not one sufficiently constant or important to be statistically significant.

A degree of tolerance of these newborn mice may be detected in a second skin homograft from the same donor. This graft does not undergo the characteristic second-set reaction at the sixth day.

The preparation of our extracts of basal epidermal cells does not present any major difficulty. The source of these extracts is practically unlimited and the antigenic activity of the extract very high.

SUMMARY

This communication explains the reasons for our preference for the basal epidermal cells in the preparation of antigenic extracts.

A first technique, where the epidermal cells were simply ground with fine glass powder, gave an extract with a very high antigenic activity, but with a prohibitive toxicity.

Two other techniques were then tried: in one of these, the epidermal cells were destroyed mechanically; in the other, chemically (by the action of trypsin).

Both components obtained by these procedures have a very characteristic antigenic activity, even with low doses, and are without toxicity even at much higher doses.

These extracts are efficient when given by all the usual routes: intraperitoneal, intravenous, and intradermal. The intradermal injection, however, at the site of the future bed of a skin homograft, produces a much more violent and rapid breakdown.

As demonstrated by the dosage of the nitrogen, the cellular destruction from the action of trypsin eliminates a part of the proteins. Thus this procedure gives an antigenic component that is perhaps more favorable to further chemical investigation. Furthermore, if there is any difference, the antigenic activity of the extract prepared with trypsin is probably the greater.

If the cell-free extracts of epidermal cells have a very high antigenic activity, they have not produced thus far any sign of total tolerance in mice injected in the first hours after birth. These mice, challenged in adult life by skin homografts of the same donor, nevertheless enjoy a certain degree of tolerance. In some cases prolonged survivals of the homografts are observed, but they are too irregular in number and duration to be considered. Large-scale investigations that will be statistically significant must be made.

Another sign of a certain degree of tolerance in these mice may be demonstrated by a second skin homograft from the same donor; this graft does not show the characteristic second-set reaction at the sixth day.

All the experiments described in this communication were performed on mice and on rabbits. The results are very similar in the two species.

The preparation of the extracts of basal epidermal cells does not present any major difficulty.

The source of the extracts is almost unlimited and their antigenic activity is very high.

References

Albert, F. & G. Lejeune-Ledant. 1958. Evolution dans nos moyens d'induction de la survie prolongée des homogreffes de peau chez l'adulte. Bull. Acad. Roy. Med. Belg. **24:** 82–112.

Albert, F. & G. Lejeune-Ledant. 1959. Prolonged survival of skin homografts in adults. *In* Biological Problems of Grafting : 110–126. A symposium. Blackwell Sci. Publ. Oxford, England.

Billingham, R. E. & P. B. Medawar. 1951. The technique of free skin grafting in mammals. J. Exptl. Biol. **28:** 385–402.

Billingham, R. E., L. Brent & P. B. Medawar. 1954. Quantitative studies on tissue transplantation. II. The origin, strength and duration of actively and adoptively acquired immunity. Proc. Roy. Soc. London. **B143:** 58–80.

Billingham, R. E., & E. M. Sparrow. 1955. The effect of prior intravenous injections of dissociated epidermal cells and blood on the survival of skin homografts in rabbits. J. Embryol. Exptl. Morphol. **3:** 265–285.

Billingham, R. E., L. Brent & P. B. Medawar. 1956. The antigenic stimulus in transplantation immunity. Nature. **178:** 514–517.

Billingham, R. E. & L. Brent. 1957. A simple method for inducing tolerance of skin homografts in mice. Transplantation Bull. **4:** 67–71.

Billingham, R. E., L. Brent & P. B. Medawar. 1958. Extraction of agents causing transplantation immunity. Transplantation Bull. **5:** 377–381.

Billingham, R. E., L. Brent, J. B. Brown & P. B. Medawar. 1959. Time of onset and duration of transplantation immunity. Transplantation Bull. **6:** 410–414.

Haskova, V. & J. Majer. 1959. The problem of the induction of immunological tolerance to grafts by non-cellular material. Folia Biol. (Prague). **5:** 1–7.

Oth, A. & A. Castermans. 1959. Study of transplantation antigens from isolated nuclei. Transplantation Bull. **6:** 418–424.

Discussion of the Paper

Merrill: Billingham and I, in talking about opening the discussion of this paper, agreed that the first thing to do would be to congratulate the authors on having carried out what those of us who have done so realize is an extremely tedious and time-consuming procedure, even more so, I gather, when one is dealing with epidermal cells rather than lymphocytes or spleen cells. However, I think the procedure is worth following because, as Lejeune and Albert have pointed out, one has in this a method by which one can travel two roads, the road of chemical investigation and, at the same time, we hope, that of biological investigation. It is important in every one of these extracts to make sure that they do have biological activity, as the authors of this paper have done.

It seems to us, certainly, that some day we shall be able to isolate a chemically defined transplant antigen, if such exists. Certainly, the first step in so doing is the sort of step that has been taken by the authors of this paper.

It may well be that perhaps there is a difference between the transplant antigen as it appears in epidermal cells and that in the lymphocyte. For years we have been taught that this probably is not true, that this reaction is individual-specific rather than tissue-specific. However, there is evidence that this may not be true. Certainly, the testis and perhaps the parathyroid act somewhat differently. One can produce immune sera against lymphocytes.

It will destroy, specifically, lymphocytes and not polys. Accordingly, there may be some difference, and this sort of attack or approach is one way in which it might be illustrated.

I wonder if the authors of this paper have observed the sort of thing that we have seen in dealing with these extracts: that when one uses a fresh whole cell, one obtains, indeed, an individual-specific reaction.

However, when one traumatizes the white cell, allows it to stand a little too long, mixes it up (particularly when subjecting it to sonication), one finds that this individual specificity is lost.

Specifically, what we have found is that the skin testing we have used is perhaps more specific when we use whole white cells and much less specific for the graft donor when we use lysate. It seems to me only natural, perhaps, that if one subjects these antigens to such trauma one might perhaps destroy more specific groups.

We have attempted to take advantage of this in the production of tolerance, much as the authors have, but have reasoned that if whole living cells were required by the neonate, perhaps—if we got the embryo young enough—we could use lysate, which is certainly less potent, milligram for milligram, and perhaps less specific.

In the rabbit we have good reason to believe that the molecular size and configuration of this molecule will permit passage by the placenta. We have attempted to produce tolerance with this by injecting the pregnant mother. Unfortunately, we have not succeeded and, if we have any results at all, they point in the other direction. In all probability we have immunized the mother rather than produced tolerance in the fetus.

However, I think, in light of the findings described in the preceding papers that one must not abandon attempts to produce tolerance with a nonviable lysate, as it may well be that persistence of living cells is not required and that, if it could be done in this fashion, it would certainly be a great advance.

Charles F. McKhann: I support the very interesting findings of Albert and Lejeune-Ledant. James Berrian and I have been studying a system of transplantation immunity involving a single weak histocompatibility gene at the H-3 locus in mice. This is considered a weak gene because first-set homografts survive 20 to 21 days, as contrasted with the usual 8 or 9 days seen in H-2 incompatibilities.

Second-set homografts in these strains of mice survive 6 to 8 days, provided the immunizing stimulus was a first-set skin homograft. When other tissues were used for immunization, considerable differences were found. We found that spleen cells and lymph nodes were completely ineffective when administered intraperitoneally. Other tissues, including bone marrow, buffy coat, and thymus cells, were somewhat more effective, permitting the graft to survive between 10 and 12 days, and renal epithelium and epidermal cells (obtained by trypsinization with magnetic agitation of fragments of whole tissue) were very effective, more effective, or at least as effective, as first-set skin grafts in immunizing the animals.

First, we thought that perhaps the spleen cells and the lymph node cells did not contain the specific antigen of this strain of mice. However, several studies show that this probably was not the case. When these same cell-type suspensions were injected subcutaneously, some marginal but demonstrable immunity could be obtained. Second, we found that if the same type of cell suspension was administered to newborn mice, long-lasting good tolerance was obtained. Third (I caution that this has not been confirmed many times; sometimes it works and sometimes it does not), when the spleen cells which, by themselves, will not normally induce immunity are treated with trypsin and injected intraperitoneally, we obtained good immunization.

Our interpretation of this is that the antigen is not lacking from these cells, but that probably the differences between these varying body tissues and their efficacy in immunization depends upon the location of the antigen within the cells and, perhaps, the way in which the cells themselves are handled within the host following injection. Perhaps trypsin itself and the action of trypsin on these cells may uncover antigens that become more accessible than they otherwise would be.

F. ALBERT: May I mention that we agree entirely with this concept, as we consider epidermal cells as having more violent antigenic properties than have other living cells, that is, spleen or lymph node cells, especially when we have had to prepare antigenic material for adequate testing.

We hope to continue our investigation because we consider it very important to have what we can call a "complete" transplantation antigen that is capable of providing both transplantation immunity and transplantation tolerance.

STUDIES ON THE HEALING OF SUCCESSFUL SKIN HOMOGRAFTS IN ALBINO RATS*

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The general features of wound healing have been explored extensively. During the present century numerous investigators, including Marchand (1901), Arey (1936), Carrel and Du Noüy (1921), Lindquist (1946), Needham (1952), Gillman *et al.* (1955–1956), Williamson (1957), and Edwards and Dunphy (1958) have published reviews of the progress in the field. Despite the various approaches in examining the problem of normal wound healing, the nature of the stimulus that prompts tissue to regenerate and to repair loss is still unclear. Even greater uncertainty surrounds the processes involved in the conditioned healing-in of such foreign tissues as homografts. A very able review of the problem by Schultz (1959) advances the concept that immunological incompatibility is the cause of the rejection of foreign tissue and it attempts to reconcile the exceptions to this theory. Schultz states that enzymological changes in the cellular nucleus and cytoplasm may act to abrogate the incompatibility of foreign tissues and permit them to be accepted by the host. However, it is evident that much more work must be done in this field.

Autografts of skin, whether split or full-thickness, are well tolerated by the individual. Skin homografts of whatever thickness, however, are invariably rejected by the normal host. Skin isografts exchanged between uniovular twins or long-term inbred animals of the same sex usually will be tolerated. From many well-documented studies it has been assumed that acceptance or rejection of skin is dependent upon conformity of genotypes. Snell and his co-workers (1953) have implicated the presence or absence of H-2 alleles as decisive in the tolerance of homografts, and Eichwald and his co-workers (1957) have added that the Y chromosome is also a potent factor. Many investigators have shown also that the genetic factors can be circumvented by the use of whole-body irradiation (Trentin, 1958), cortisone treatment (Toolan, 1954, 1958), pretreatment with donor cells (Medawar, 1954), and homografting during the early post-partum period (Medawar and Woodruff, 1958), and by concomitant treatment with pooled serum fractions containing alpha globulins (Kamrin, 1958*a*, 1958*b*, 1959).

There are numerous aspects to be considered in even the short-term survival of skin homografts. One feature that has occupied many investigators is the role played by the skin (hair) cycle in skin homografts. Recent work by Randall and Dushoff (1958) suggested that homografting during the telogen skin cycle is more conducive to prolonged retention. Ballantyne and Converse (1957) demonstrated that homografting in the rat during the earlier catagen skin cycle brought about the longest survival time. Woodruff and Simpson (1955) used split-thickness skin homografts and obtained the same range of survival irrespective of the hair cycle. Using irradiation techniques, Hardin

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and Werder (1955) obtained 1-year survival of homografted mouse skin; there was no correlation of acceptance with the state of the skin cycle. My own detailed examination of the data of successful skin homografts failed to disclose any correlation between the various skin cycles and subsequent tolerance of the grafts.

Another aspect examined by several of the investigators cited above was the presence or absence of the panniculus carnosus. It would appear that a homograft without a panniculus had the best chance for prolonged survival. Other workers, for example, Ballantyne and Converse (1959) and Hughes and Dann (1941), also have examined the effect of aging and of the vascularity of the skin.

A large volume of work has been devoted to examining the various systemic events that occur during wound healing. Pertinent to this work are the investigations of Gjessing and Chanutin (1947) on the plasma changes with injury, Roth's (1954) research on protein production in the thermally injured rat, and the effects of the protein diet on the serum albumin (Jeffay and Winzler, 1958). Kamrin (1958*a*) showed that the retention of a normal albumin/globulin ratio was a normal accompaniment of successfully parabiosed rats.

The present investigation of the sequence of events that occur in the union of the homograft with the host was possible through the achievement of frequent successful skin homografting. This report therefore will describe the four following aspects of the problem: (1) total growth (weight increases) of normal animals, successfully homografted recipients and unsuccessfully homografted recipients; (2) planimetry measurements of the growth of successful homografts; (3) histological changes at the junctional area of the apposed tissues and within the successful homograft during its integration into the host; and (4) the changes that occur in the blood serum of the various treated recipient animals and controls.

Materials and Methods

Wistar pen-bred rats were used as the subjects of this experiment. All litters were kept together after weaning until paired with a nonlittermate for reciprocal homograft exchange. The paired rats (25 to 30 days of age) were usually of the same sex, but not necessarily of the same age or hair cycle. Accurate records were maintained of the mothers to prevent inbreeding or close kinship.

Under ether anesthesia the adjacent abdominal area hair was removed with electric clippers from each of the animals while they lay side by side. Sections of skin of approximately the same size (2.5 cm. × 2.5 cm.) were cut from each animal's abdominal wall, rotated 180°, and reciprocally exchanged. The full-thickness graft usually had the panniculus carnosus adherent to it. Interrupted cotton sutures were used to sew the graft closely to the margins of the host tissue, so that both tissues were tightly approximated to each other. A gauze pad was placed over the entire area, followed by a small thick pad adapted to the center of the graft. Adhesive tape was used to secure the gauze pads firmly to the area and to assure close contact between graft and host.

Pooled rat serum fractions containing alpha globulins were injected intraperitoneally at the termination of the operative procedures. The lyophilized globulins were dissolved in 0.15 *M* NaCl and administered so that 0.5 to 1.0 mg. globulin was given for each 10 gm. of animal weight. The lyophilized and dissolved material was stored at 7° C. and, when it was used for injections, it was kept cold in beakers containing cracked ice. Reinjection of the animals with the same material was made on postoperative days 3, 6, 9, and 12.

Measurement of the surface area of the graft was made by placing a rigid strip of Plexiglas over the area and tracing the outline with a soft lead pencil. This tracing was transferred to paper for permanent record. Using a polar planimeter,* the outline was measured and recorded as square centimeters of surface area. Statistical analysis of similar data by Lindquist (1946) indicated that the error increases inversely to the size of the area. Multiple determinations showed that measurements of 3.0-sq. cm. areas resulted in less than 1.0 per cent error, while similar examination of 0.1 to 0.25 sq. cm. areas elicited 3.0 to 8.0 per cent errors. The present series of measurements, which I made every 3 to 5 days, showed that the individual variations tended to cancel out with the establishment of definite trends.

The animals were weighed every second day, and the results were recorded carefully. The operated areas were examined visually at the same time, and any major changes such as drying, hardening, contracting, desquamation, depilation, blanching, redness, necrotic areas, sloughing, or excellent union (with or without new hair growth) were noted. All animals were kept under the same environmental and nutritional conditions.

Biopsies of the junctional area were taken daily from a series of animals and used as an example of healing if the graft was fully tolerated and became a healthy integral part of the host. Such uncertainty required that biopsies from 4 animals be made daily during the first 6 days. By day 7 the apparently successful grafts were evident, and biopsies were taken from only 2 animals daily until day 12. Subsequently biopsies were taken from successful graft/host union at 2- to 10-day intervals until postoperative day 300. All biopsied tissue was fixed in 10 per cent formalin immediately after removal, embedded in paraffin, sectioned at 4 or 5 μ, and stained with hematoxylin and eosin, Giemsa, Mallory, or Bodian formulas.

Blood was drawn from the external jugular vein (1.0 ml.), permitted to clot at room temperature, and spun in a refrigerated centrifuge at 2500 rpm for 10 min. The resulting serum, after determination of specific gravity, was dialyzed against Veronal buffer, *p*H 8.6, ionic strength 0.10 for 90 to 120 min., diluted to approximately 2.5 gm. per cent with fresh Veronal buffer, and placed in a cell for electrophoretic analysis by the Antweiler (1952) microelectrophoresis apparatus. Both schlieren photographs and interference readings were made, and the percentages of components present were calculated.

Observations and Results

Total growth. Although several hundred animals were used in the various procedures, the growth curve shown in FIGURE 1 reports the results of consistently weighing about 70 animals. From these data there appears to be a distinct sex difference in the response of the animals to the injections and operations performed. The actual curves were constructed from the means of the various individual animals weighed at the same age and do not show the standard error. Using the mean weight increase as a frame of reference, one notes that up to day 65 of age the average untreated male animal increases in weight more rapidly than the injected animal bearing either successful or unsuccessful homografts. At this time the average successfully homografted male animal of the same age is about 40 days postoperative; after this time these animals show a somewhat greater increase than the controls. The male animals that slough the homograft, usually by the postoperative day 15, show a consistently diminished growth curve.

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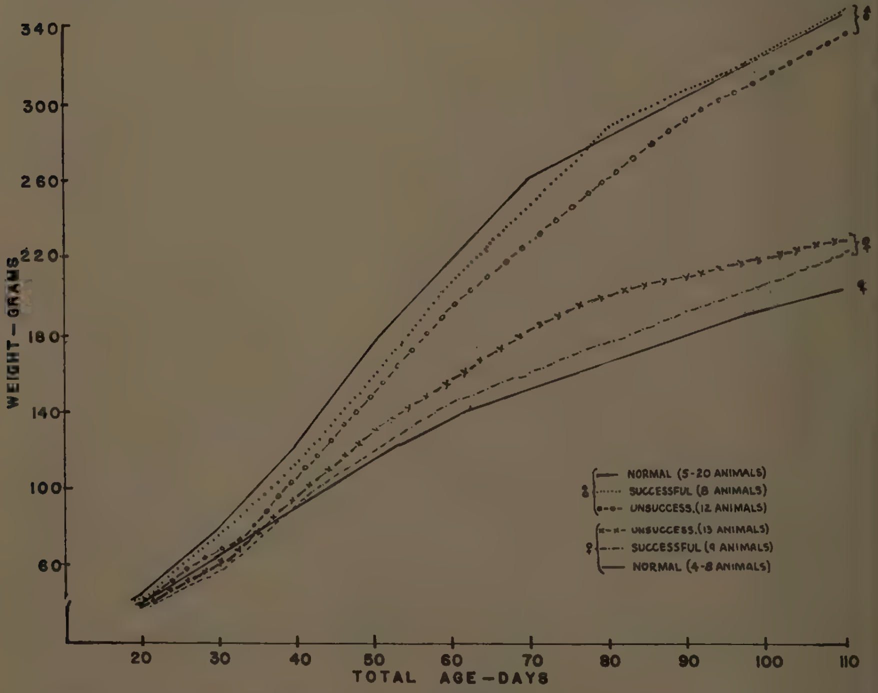

FIGURE 1. The greatest divergence of daily weight increments in both male and female normal and successfully homografted albino rats after day 55 of age. Marked divergence of the growth curves of male and female unsuccessfully homografted animals does not occur until day 80 of age. The 25- to 30-day differential in growth response can be attributed directly to the period required to heal the wound remaining after the homograft has sloughed.

Female animals show a somewhat different pattern. After an initial higher growth rate than the experimental animals (35 to 40 days of age) the controls show lesser daily increases in weight. After an early postoperative depression of the weight, animals sloughing unsuccessful grafts show the highest increase in weight. The successfully homografted animals are intermediate between the controls and the unsuccessful experimental animals.

Growth of homografts. Removal of the skin with the panniculus carnosus usually results in a large defect on the abdominal wall. When the graft is sewed into the defect area the host tissues adapt themselves to the graft margins, making a close seal.

Numerous measurements of the size of the graft at the time of transplantation show that it varies from 6.0 to 6.5 sq. cm. Routine measurements were made when the protective dressing was removed on postoperative days 5 or 6 and subsequently at 2- or 5-day intervals for the first 100 days.

Unsuccessful grafts usually show drying and blanching at the time when the dressing is removed. Such grafts have shrunk somewhat in size, but this shrinkage is accelerated after day 6, so that by the time it is sloughed at about day 15 the graft will have an area of about 1.0 sq. cm.

Successful homografts show a sex difference during the early contraction phase. Female rats bearing successful female skin homografts will demonstrate their maximum contraction to approximately one half of the original size by the day 21. Contraction of the male skin homograft on the male will show a lesser diminution of surface area and will not reach its maximum contraction until the day 28. After this period of integration into the host's body economy, growth in surface area of the homograft will increase slowly consistent with the over-all growth of the particular sex. FIGURE 2 illustrates the progress made by 4 male and 3 female animals bearing successful homografts for periods of 200 days. The shrinkage period appears to be wholly dependent upon the sex of the animal. Subsequently the increase in surface area is dependent upon the over-all size of the particular animal bearing the successful graft.

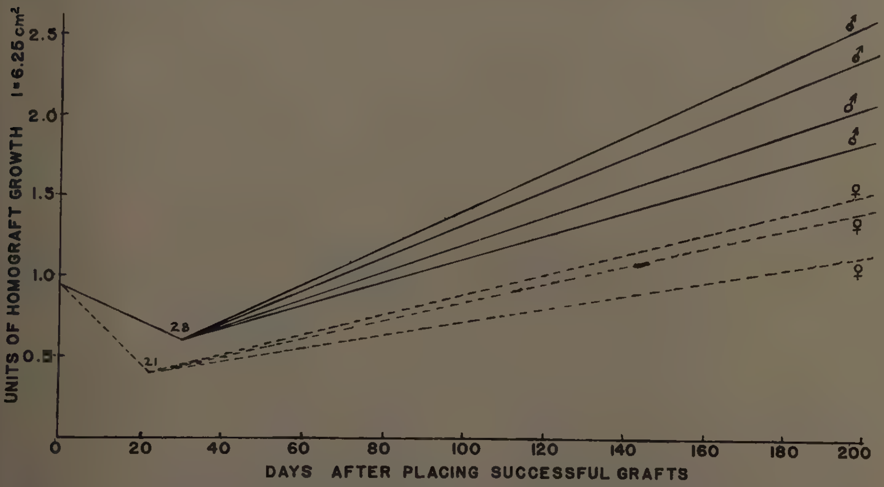

FIGURE 2. Representative changes in the surface area of successful homografts. Detailed studies of planimetry measurements made of the successful homografts from postoperative day 5 demonstrate that the initial contraction of the total surface area is characteristic for each sex. Subsequent growth of the homograft is individualistic; that is, dependent upon the growth (weight) potential of the recipient.

Histological examination. Histological examination of autografted skin showed that the removal and replacement of the tissue to the same site by suturing resulted in minor connective tissue junctional inflammatory changes during the first 6 days. Subsequently there was uneventful healing. The included panniculus carnosus showed major changes, with widespread loss of cross striations on day 3, some fragmentation of the muscle fibers, and fibroblastic infiltration by day 6. Hair growth of the autograft resumed on days 6 to 10.

Examination of normal skin from abdominal wall at 13 days post-partum to 75 days of age shows that:

(1) The epithelial cells increase in total size from 0.005 mm. to 0.01 mm. The epithelial lining of the skin is 1 to 2 cells in thickness at 13 days and increases to about 2 to 4 cells in thickness at 75 days of age. The epithelial nuclei are elongated at 13 days and round or oblong at 75 days.

(2) FIGURE 3 shows that the underlying dermis, measured between the epithelial layer and the panniculus carnosus, has a total thickness of 0.13 mm. at 13 days and a maximum of 0.7 mm. at 75 days of age. The amount of fat cells present fluctuates from one third to two thirds of total thickness.

(3) Only a small number of erector pili muscle fibers is present in the skin of male and female rats. Although sparse, an apparently large number is seen in the male skin.

(4) The panniculus carnosus of the 13-day-old skin measures 0.01 to 0.015 mm. in thickness and is made up of several fibers. Cross striations are evident. The number of bundles increases in size and thickness so that the skin of a 75-day-old rat has a panniculus measuring between 0.2 to 0.35 mm. in diameter.

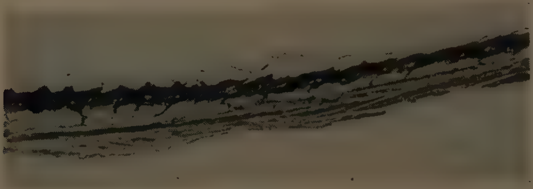

FIGURE 3. Normal skin from the abdominal wall of a 43-day-old female rat. ×6.

Initial daily examination of the healing of skin homografts showed the following major changes: the graft tissues are more deeply staining than the host and show greater condensation of the connective tissues. Also, the cells making up the hair follicles and sebaceous glands are denser and smaller, and appear to have lost much of their cytoplasm. The epithelial surface of the graft shows early loss of its inherent undulating character (corrugations). This characteristic is retained for several months and differentiates it from the normal folds of the host skin.

Postoperative day 1. The gap between the host and graft margins varies in width between 0.03 to 0.2 mm (FIGURES 4 and 5). It is loosely filled with cellular debris and fibrin of clotted blood. The host epithelium adjacent to the graft margin has migrated in a thicker layer than that overlying the rest of the host skin, so that it covers the cut reticular surface of the host and has sent an extension of epithelial cells into the gap toward the connective tissues of the graft. This bar of epithelium lying under the clot appears to be unsupported by any tissue and probably rests on the accumulated extravasated serum in the area.

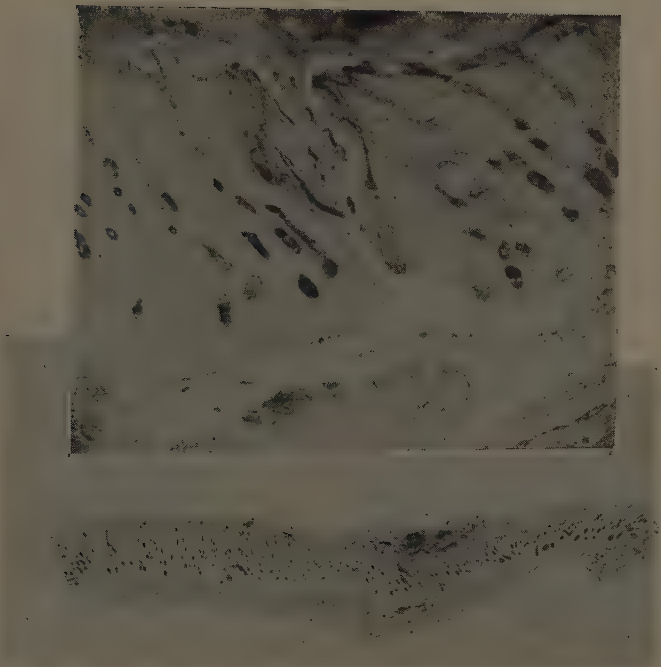

Figure 4. Postoperative day 1. Upper enlargement (×42) shows outward growth of epithelial bar from host (*right*), along its reticular layer and then toward the graft (*left*). Lower inset (×6) shows complete biopsy section. (Recipient, 24-day-old female; donor skin, 25-day-old female.)

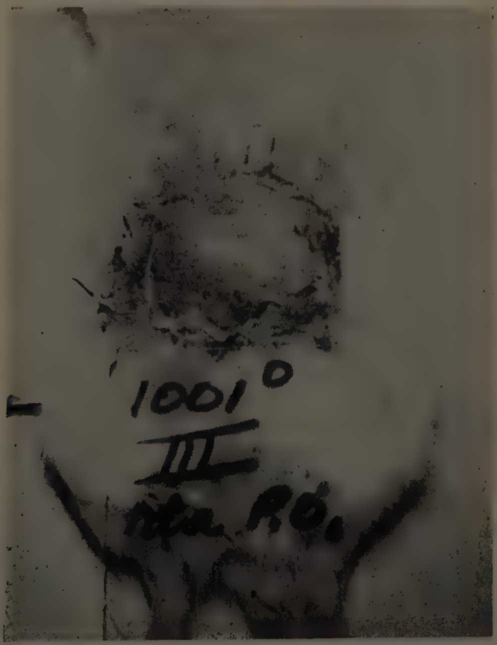

Figure 5. Appearance of graft on host at end of first 24 hours. Note the presence of some peripheral engorgement in the area adjacent to the suture line. The notation III refers to treatment with pooled rat serum fraction containing 17.0 per cent alpha globulin, 66.8 per cent beta globulin, and 12.2 per cent gamma globulin.

Epithelium of the graft adjacent to the cut surface has been lost, together with some of the connective tissue elements. All sebaceous gland cells in the immediate vicinity of the cut have disappeared, but gland cells further away show cytoplasmic loss and pyknotic nuclei. The fibers making up the panniculus carnosus appear to be ruptured by swelling, although the individual muscle bundles appear smaller in diameter. Numerous dilated capillaries and blood vessels filled with blood cells are seen distributed throughout the graft.

Postoperative day 2. The gap between the tissues has diminished. The epithelized lips of the wound (host and graft margins) are deeply inverted toward the connective tissues. The previously seen bar of epithelial cells from the host has contacted the graft connective tissue and is now burrowing its way into the dying tissue of the graft. The area below the bar is now seen to have a few free-lying red blood cells. Actual contact has now been made between the host dermis and that of the graft at the level of the superficial fascia of the host.

The graft adjacent to the junctional area shows greater destruction of the epithelial and connective tissue elements. Massive round cell infiltration has not as yet begun in the graft, but an increase of such infiltration is seen in the host's marginal tissues. Sebaceous gland cells are infrequently seen in the graft and, where present, the cells are small and consist almost entirely of nuclei. Hair follicle cells have become clumps of pyknotic nuclei, with barely perceptible cell outlines. The fibers of the panniculus carnosus in the graft and near the margin of incision on the host show greater disruption and loss of cross striations. Many of the muscular nuclei have disappeared.

Postoperative day 3. The host epithelium has contacted and partially covered the graft (FIGURES 6 to 7). Epithelial cells have migrated to a point near the junction of the tissues to a depth of 6 to 10 cells for a distance of about 0.5 mm. This contrasts with the average thickness of the host epithelium layer, which is usually 2 or 3 cells deep. Where the suture has entered the host tissue, there is a heaping up and ingrowth of the epithelial cells along the track.

The entire host margin has approximated itself to the graft so that no actual gap exists between the tissues. However, the junctional margins of the host and graft tissues are still inverted so that a **V** is formed. Nerve fibers stop at the incision area of the host.

Epithelial cells remaining on the graft surface have become condensed. Within 0.7 mm. of the cut area many of the cells have been sloughed off into the covering blood clot. Beyond this distance the epithelial cells are vacuolated with light-staining nuclei. The underlying stratum reticulare is compressed and densely staining.

There are no signs of sebaceous gland cells in the graft, and the hair follicle cells appear as mere accretions around an old hair shaft. The fat cells in the submucosa of the graft appear somewhat compressed and less in volume than those seen previously in this region or in the host. This results in the panniculus carnosus lying closer to the surface than in the host tissue. The muscle fibers appear more fragmented and amorphous in some areas with complete absence of cross striations and nuclei.

There is a greater accumulation of mast cells and small lymphocytes in the host than in the graft.

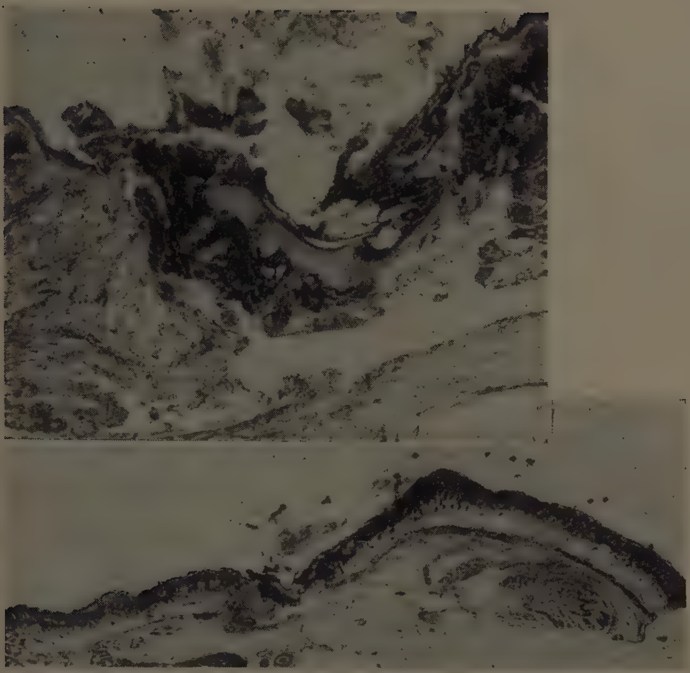

FIGURE 6. Postoperative day 3. Upper enlargement (×42) demonstrates an increasing accumulation of epithelial cells burrowing into necrotic tissue of graft (*left*). Note the increased staining intensity of the host (*right*) tissues adjacent to incision area. This is more clearly seen in the lower section (×6). Recipient (female) aged 27 days; donor skin (female) aged 28 days.

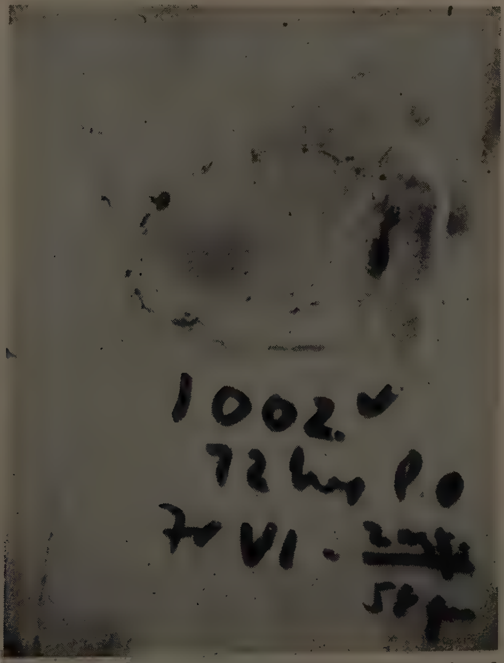

FIGURE 7. Appearance of graft at the end of postoperative day 3. Engorgement has subsided. Dark areas indicate bleeding where sutures were dislodged during the removal of dry dressing. The notation VI refers to treatment with pooled rat serum fraction containing 17.0 per cent alpha$_1$ globulin, and 83.0 per cent alpha$_2$ globulin.

Postoperative day 4. At this time there is excellent union of graft with the host. The **V**-shaped inversion of tissues at the junction point is still present and now measures about 0.2 mm. in depth and 0.1 mm. in width. The large accumulation of epithelial cells in the junctional area has diminished and spread to a lesser thickness to contact the graft epithelium. No gap now exists beneath the epithelial bridge, and some granulation tissue has made its appearance.

Capillaries have grown into the graft, as the previously engorged vessels that contained crenated blood cells are now empty. With the influx of capillaries there is evidence of an increased infiltration of mast cells and lymphocytes.

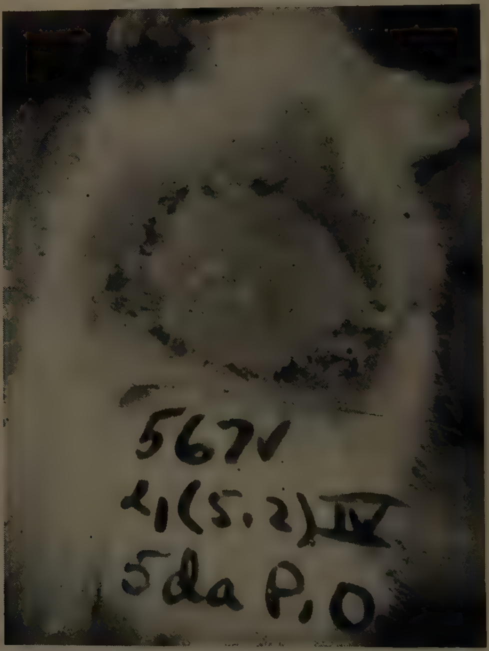

FIGURE 8. Appearance on postoperative day 5, showing the merging of graft periphery with that of the host tissues. The homograft has the same color and softness as the host skin. The notation α_1 (5.2) IV refers to treatment with an electroconvection product derived from pooled rat serum fraction IV at *p*H 5.2. This material contains 76.1 per cent alpha_1 globulin, 8.3 per cent alpha_2 globulin, and 15.6 per cent beta globulin.

In the junctional area and extending over on to the graft the epithelial cells are piled up about 6 to 10 layers deep. For the first time downgrowths of epithelial spurs are seen that may presage the position of future hair follicles and sebaceous gland cells. The previously formed hair follicles in the graft have now become discrete accumulations of nuclei; no sebaceous gland cells are seen in the tissue.

Postoperative day 5. A very thick shelf of epithelium covers the gap between the tissues, almost completely eliminating the previous **V** cleft; in its place there is a broad depression measuring 0.25 mm. wide by 0.5 mm. deep (FIGURE 8). Despite the growth of granulation tissue,.a small empty area is still present under the bar of connecting epithelium.

The continuous covering of junctional epithelium has the characteristic appearance of the epithelium covering the host. The distant epithelial cells on the graft are plumper than formerly, and the round nuclei stain almost normally. There are initial signs of recognizable regenerating (?) sebaceous gland cells within the graft. However, the fat cells adjacent to the panniculus have almost entirely disappeared from both graft and host in the region adjacent to the incision.

Numerous capillaries have established continuity with the host, so that few cells remain in the vessels. The appearance of parallel-lying fibroblasts in the junctional area probably indicates the site of the ingrowing capillaries.

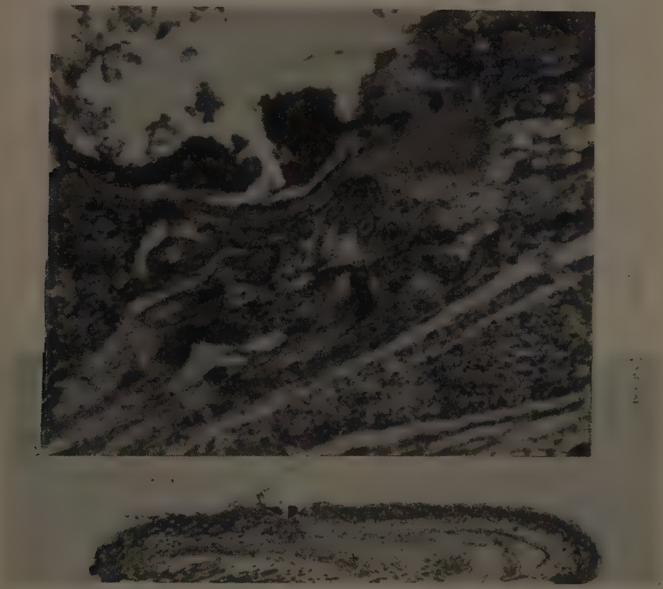

FIGURE 9. Postoperative day 6. Upper enlargement of junctional area (×42) shows complete contacting of host epithelium (*right*) with graft epithelium (*left*). The heaping up of epithelium appears to serve as a reservoir for subsequent migration. Lower section (×6) shows the continued presence of the panniculus carnosus and the suprapannicular fatty layer. Inflammatory reactivity has diminished, and granulation tissue is evident. Recipient (female) aged 30 days; donor skin (female) aged 31 days.

The muscle fibers appear to be distorted, disrupted, and amorphous in some areas, with no visible cross striations. The muscle bundles are infiltrated with fibroblasts, collagen fibers, and free blood cells.

Postoperative day 6. To a great extent the deep original **V**-shaped cleft has been flattened out (FIGURE 9). The epithelium overlying the junctional area is still multilayered and has longer spurs growing into the submucosa. The host tissue at this time appears normal except close to the line of union, where there is a thick deposit of fibrin and collagen fibers. Many more capillaries are seen.

Within the graft proper the previously quiescent hair follicle cells appear to regain their cytoplasmic content, and the sebaceous glands are also evident. There is an infrequent appearance of fat cells in the deep subcutaneous tissues of the graft. The muscle fibers of the graft panniculus carnosus are still abnormal in staining and show no evidence of cross striations. However, the muscle nuclei appear to be more normal.

Postoperative day 7. In addition to almost complete subsidence of inflammatory signs in the host, the junctional epithelium has decreased in thickness adjacent to the area of union (FIGURE 10). About 0.3 mm. from the line of union the epithelial area over the host resumes its normal thickness and is marked by the presence of fully functional hair follicles and sebaceous glands. The tissues marking the line of union have smoothed out to a great extent and show only a depression of 0.35 mm.

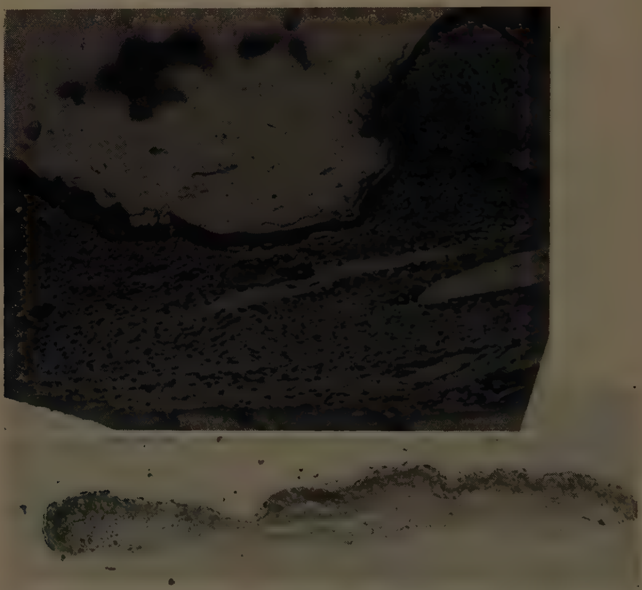

FIGURE 10. Postoperative day 7. Complete union of tissues of homograft and host is seen in both upper (×42) and lower (×6) micrographs. Host tissue is at left, graft tissue is at right. Note the diminution of epithelial accumulation and decrease in fatty layer. The junctional area is still visible as a cleft between host and graft. Recipient (female) aged 31 days; donor skin (female) aged 32 days.

The accumulation of epithelium has migrated further on to the graft, as there is a thick layer (4 to 6 cells in depth) 1.7 mm. from the line of union. Most of the hair follicles and sebaceous glands have attained a normal appearance, and there are definite signs that the severed regenerating nerve fibers are following the epithelial cells into the graft. The muscle layer appears to be somewhat attenuated in volume.

Postoperative day 8. Reaction to the presence of the graft has diminished to the point where the hair follicles of the host are found within 0.08 mm. of the line of union. The epithelial covering over the junctional area has diminished in thickness and extends only 0.15 mm. to either side of it. The underlying granulation tissue in the junctional area is now arranged in parallel layers, with concomitant leveling of the line of union so that only a 0.1-mm. inverted area remains.

The thickened graft epithelium shows a few epithelial pegs or spurs. Although the connective tissues of the graft appear to have a different staining intensity, all the elements appear to be returning to normal. Fat cells are seen to be forming again about the remaining panniculus carnosus, while the muscular fasciculi themselves are thin and compressed, with no cross striation.

Postoperative day 9. The line of union between host and graft has been smoothed out so that no inversion of tissue is seen (FIGURE 11). The epithelial layers in this region are still rather thick and show some peg formation. The thick epithelial layer extends only for 0.1 mm. from the line of junction. The host tissues have shown excellent reconstruction, with elimination of inflammatory products. The various hair follicles and sebaceous glands closely occupy areas near the incision area. Within the host tissue the panniculus carnosus near the junction has become revitalized and can be seen to have cross striations.

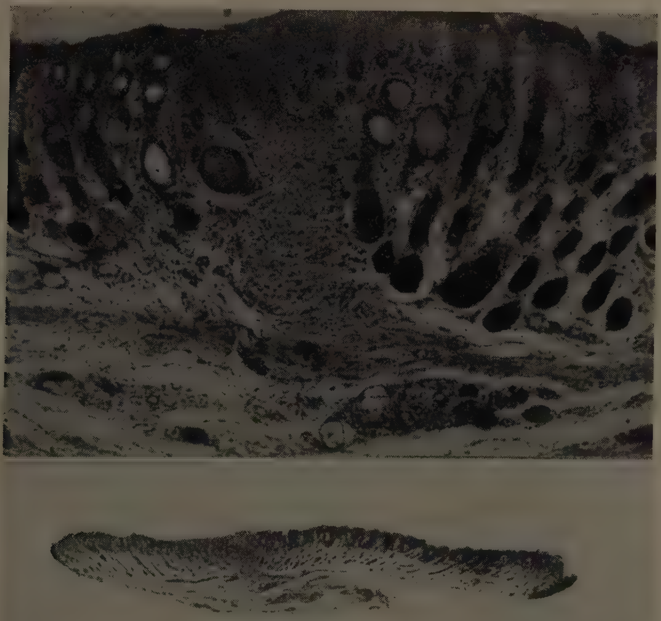

FIGURE 11. Postoperative day 9. In both lower micrograph (×6) and upper micrograph (×42) it is apparent that full tolerance of the graft (*left*) has been achieved. Note the numerous spurs projecting from the thickened layer of epithelium in the junctional area. The presence of the sebaceous gland cells in the graft is further evidence that the homograft is wholly accepted. Recipient (female) aged 33 days; donor skin (female) aged 34 days.

The thickened epithelial layers (10 to 15 cells deep) have decreased from the previous extent on the graft to only 1.3 mm. from the line of union. Spurs or pegs of epithelial cells extend from the surface epithelium into the subcutaneous area. Many hairs can be seen to be growing from the hair follicles, with concomitant enlargement of the adjacent-lying sebaceous gland cells. There is increased fat cell formation or partial reconstitution in the area underlying the hair follicles and superficial to the panniculus carnosus. The panniculus itself appears to have become thinner and compressed, so that it is now but 0.03 mm. in width and is infiltrated with numerous fibroblasts and small capillaries. There are numerous elongated nuclei present on the muscle fibers, but no cross striations.

A heavy plexus of nerve fibers has followed the epithelium into the graft, with branches to the hair follicles and glands. No connection has been made to the panniculus carnosus except for some parallel fibers lying around the periphery of the muscle bundle. Many degenerating fibers are seen among the bundles.

Postoperative day 10. The junctional area between host and graft is now perfectly smooth. The area itself is clearly demarcated by the corrugations on the surface of the host skin and the relative smoothness of the graft skin surface. The epithelial thickening is still present. There is a randomized rearrangement of the previous parallel-lying connective tissue in the junctional area. Hair follicles and sebaceous glands are now found within 0.06 mm. of the line of union.

Due to the initiation of a hair cycle in the graft, the elongating hair follicles have pushed the remnants of the panniculus carnosus further away from the outer surface. The muscle fibers in the graft show complete lysis for a distance of 5.0 mm. from the line of junction with the host. The remaining muscle fasciculi are normal-staining and have elongated nuclei, but show no striations.

The nerve fibers have formed a plexus along the host's incision area and have sent fibers along the superficial fascia and the stratum germitivum of the epithelial layer. There is a slight increase in fat cells.

Postoperative day 11. The line of union has become poorly defined (FIGURE 12). However, the absence of corrugations on the surface of the graft makes it distinctive. The epithelial covering has become more uniform over both host and graft.

There is only occasional spur or peg formation from the thinning junctional epithelium. Deposition of fat cells has increased in both the superficial and deep areas about the panniculus carnosus. The muscle fibers are still as fragmented in appearance as previously, although an occasional cross striation may be seen. The remaining muscle bundles are surrounded by parallel-lying fibroblasts and collagen fibers, which in turn are perforated by numerous small capillaries and fibroblasts. Many muscle fibers appear amorphous, despite the presence of numerous nuclei.

The nerve fibers from the host appear to have grown down about 1.0 mm. into the graft adjacent to the panniculus carnosus but not connected with it; the nerve fibers appear to underlie the entire epithelial layer.

Postoperative day 12. The most noteworthy feature of this period is the resumption of the normal appearance of the graft, with the exception that the corrugations are still largely absent from its surface (FIGURES 13 and 14). Although much of the panniculus appears to have disintegrated, the remnants show indistinct cross striations. Fat cell deposition has continued so that the panniculus carnosus is separated more from the epithelial layer. Further regeneration of the nerve fibers has occurred so that fibers can now be traced about 1.1 mm. into the graft from the host incision line.

Postoperative days 15 to 20. Microscopically, there is little change in the graft tissues except that the thin remnants of the panniculus carnosus show more marked cross striations (FIGURES 15, 16, and 17). The host tissues are restored completely to normal appearance, with the minor exception that the panniculus has been lysed for a distance of 0.5 mm. from the line of junction with the graft. It is still evident that the graft surface is relatively smooth and uncorrugated.

Postoperative days 20 to 30. Relatively little change has occurred in the histological appearance of the tissues (FIGURE 18). There are initial signs of increasing tonus of the graft skin, which on the whole is relatively uncorrugated (FIGURE 19*a*, *b*).

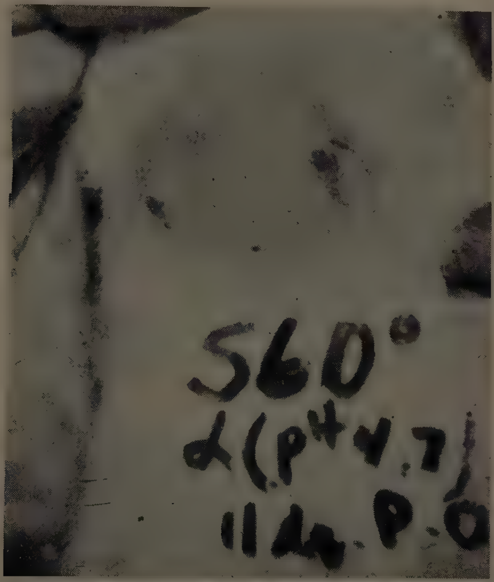

FIGURE 12. Postoperative day 11. Photograph of a 33-day-old male recipient bearing a graft from a 31-day-old (at time of transplantation) male donor. The notation α (*p*H 4.7) refers to injection material obtained by electroconvection separation at *p*H 4.7 of pooled serum fraction V. This subfraction contains 33.3 per cent alpha$_1$ globulin, and 66.7 per cent alpha$_2$ globulin.

Postoperative days 32 to 50. The graft has become an integral part of the host skin, showing a greater degree of surface corrugations (FIGURES 19*c* and 20). There appears to be more loss of the panniculus carnosus fibers; the remaining fibers seen show cross striations. Fat cells have not yet regenerated to a normal degree.

Postoperative days 60 to 400. This period is marked by almost complete restoration of the normality of the graft tissues (FIGURES 21 to 24). The distinguishing feature is the persistent diminution in the deposition of suprapannicular fat cells in the graft (FIGURE 22*b*).

Electrophoretic findings. Analyses of the blood serum of the various controls and experimental animals disclose that the globulin injections result in signifi-

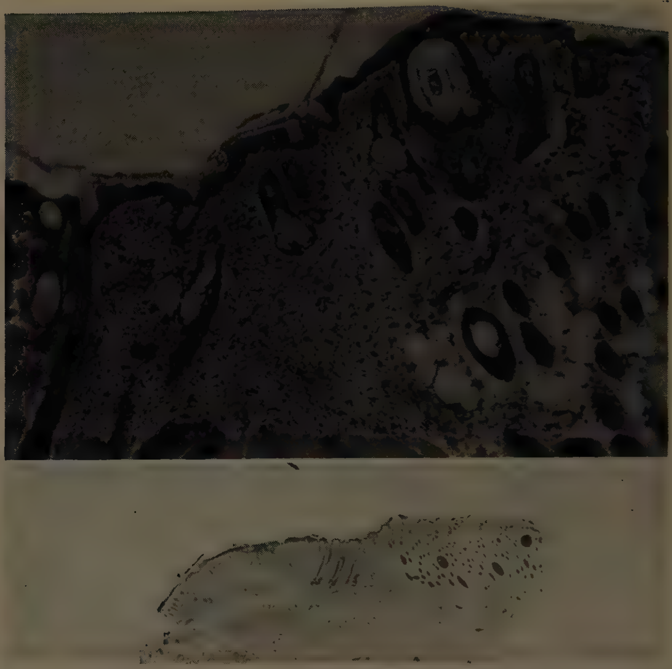

FIGURE 13. Postoperative day 12. In the junctional area between graft (*left*) and host (*right*) hair follicles are visible. The earlier presence of epithelial spurs in this area suggests that they were forerunners of these structures. Note the uncorrugated appearance of the graft surface and the absence of panniculus carnosus and fat cells. Recipient (female) 36 days old; donor skin (female) 37 days old.

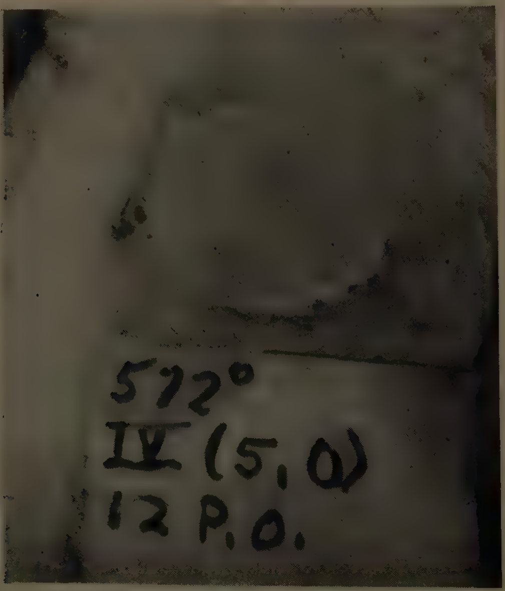

FIGURE 14. Postoperative day 12. Almost complete integration of homograft into skin of host. Excellent hair growth of a finer texture than that covering the host is present. The notation IV (5.0) refers to injection material obtained by electroconvection separation at *p*H 5.0 of pooled rat serum fraction IV. It contains alpha$_1$ globulin 26.6 per cent, alpha$_2$ globulin 57.7 per cent, and beta globulin 15.7 per cent.

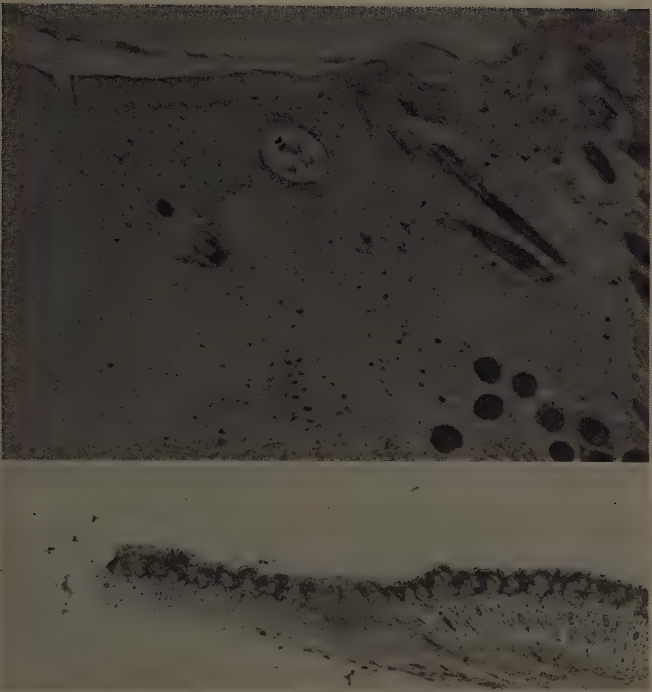

FIGURE 15. Postoperative day 15. *Upper section:* note the decrease in thickness of the epithelial covering in the junctional area (×42). *Lower section:* note the difference in appearance between the homograft (*left*) and the host (*right*), accentuated by the different hair cycles and lack of a homograft layer. Recipient (male) aged 22 days; donor skin (male) aged 35 days.

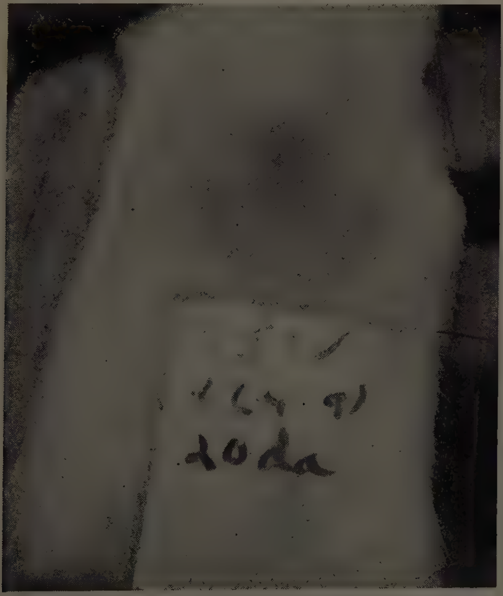

FIGURE 16. Postoperative day 20. Reversed position of homograft shows the extensive growth of hair in less than 3 weeks *in situ.* Male recipient was 25 days old at surgery and received skin from a 22-day-old donor. The notation α (4.9) refers to a subfraction from pooled rat serum fraction VI, which contained 40 per cent alpha$_1$ and 60 per cent alpha$_2$ globulins.

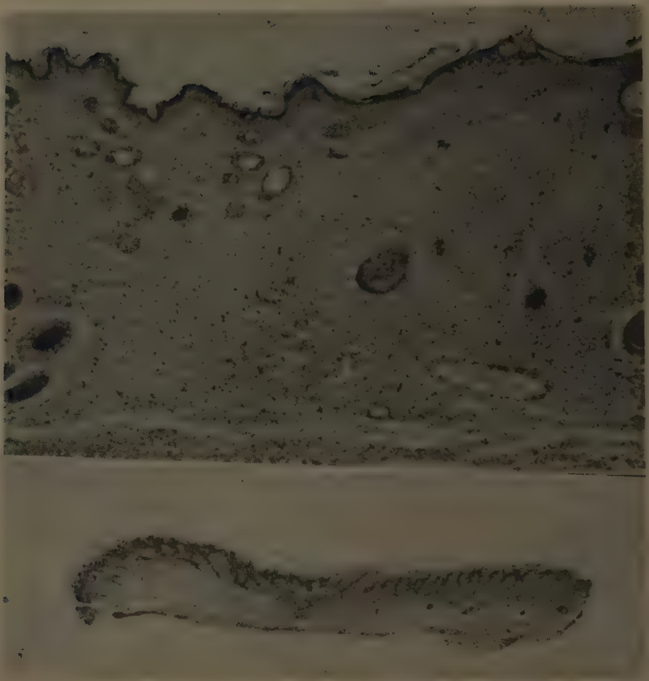

Figure 17. Postoperative day 20. Host on left and homograft on right of junctional area. New spur formation is visible here also. The granulation tissue in the area of union has become reorganized and appears uniform with the other connective tissues (*top* ×42; *bottom* ×6). Recipient (female) aged 32 days; donor skin (female) aged 33 days.

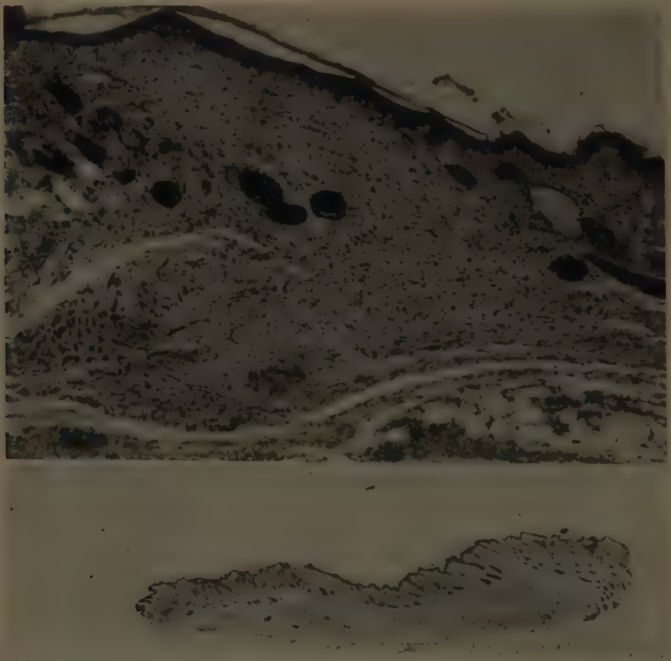

Figure 18. Postoperative day 22. More uniform appearance of the epithelial layer in both host (*left*) and graft (*right*). Spurs are no longer seen, but the fat cells are reappearing in isolated areas of the homograft. Recipient (female) aged 23 days; donor skin (female) aged 23 days.

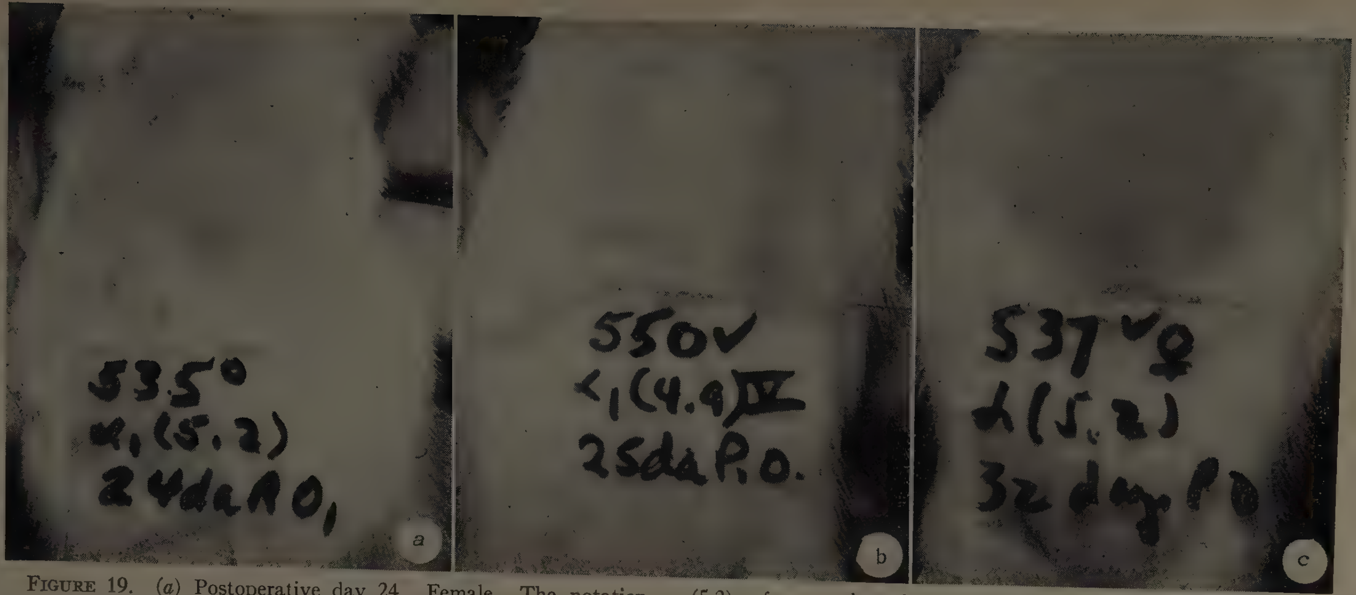

FIGURE 19. (*a*) Postoperative day 24. Female. The notation α_1 (5.2) refers to the subfractionation of pooled rat serum fraction V at *p*H 5.2 by electroconvection methods. This material contains albumin 83.5 per cent, and alpha$_1$ and alpha$_2$ globulins 16.5 per cent.

(*b*) Postoperative day 25. Male. The notation α_1 (4.9) V refers to the subfractionation of pooled rat serum fraction V at *p*H 4.9. By electrophoresis this was found to contain 59.1 per cent alpha$_1$ globulin and 40.9 per cent alpha$_2$ globulin.

(*c*) Postoperative day 32. Female. The notation α (5.2) refers to the subfractionation of pooled rat serum fraction V at *p*H 5.2, which has the same components as that listed in the notation for (*a*).

cant changes in the albumin and beta globulin fractions. Examination of FIGURE 25 clearly demonstrates that animals receiving alpha globulins only, and those receiving alpha globulins and bearing successful homografts have an increasing titer of albumin in their blood serum. This evident increase manifests itself for the first 60 to 70 postoperative days, after which the titers are reversed. It should be noted that animals that receive no treatment and slough their grafts (unsuccessful) have a lower percentage of albumin than do the normal controls or experimental animals. Again, there is a reversal of this finding at approximately 60 days postoperative.

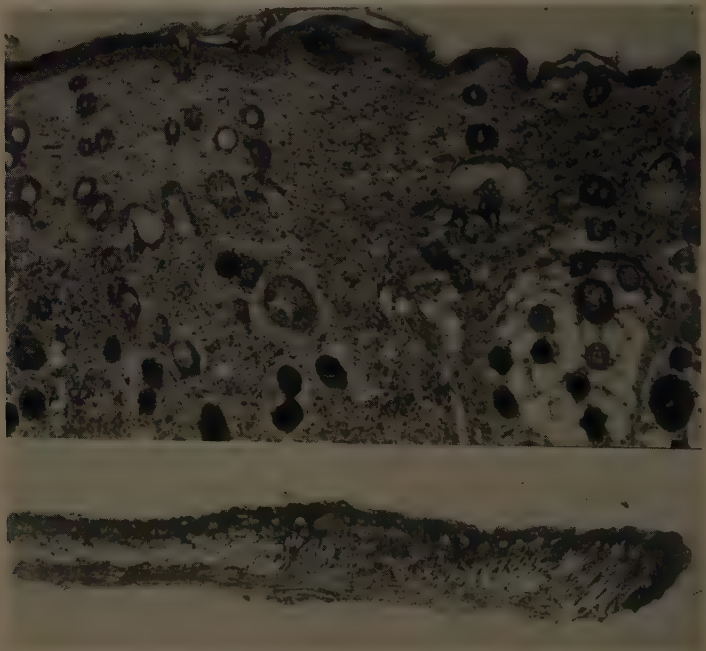

FIGURE 20. Postoperative day 38. The junctional area has decreased to about 0.3 mm. There has been little increase in the suprapannicular fatty layer. Recipient (female) aged 23 days; donor skin from female aged 24 days.

Accompanying the increase in the albumin component there is a decrease in the beta globulin moieties in all the experimental animals. Again one notes that there is a close parallel between the pattern seen in the controls injected with alpha globulin alone and the experimental animals subjected to the alpha globulin injections and successfully homografted.

Despite the injection of the alpha globulins (a total of approximately 50 mg. over a 12-day period) and the additional stimulation of a skin homograft, there are no significant changes in the alpha globulin or gamma globulin titers. In the normal animal, irrespective of sex, the average percentage of alpha$_2$ globulin is 4.0 to 4.8 and of gamma globulin 8.0 to 12.0. Changes in the gamma globulin titers may be found if the sloughing graft becomes infected.

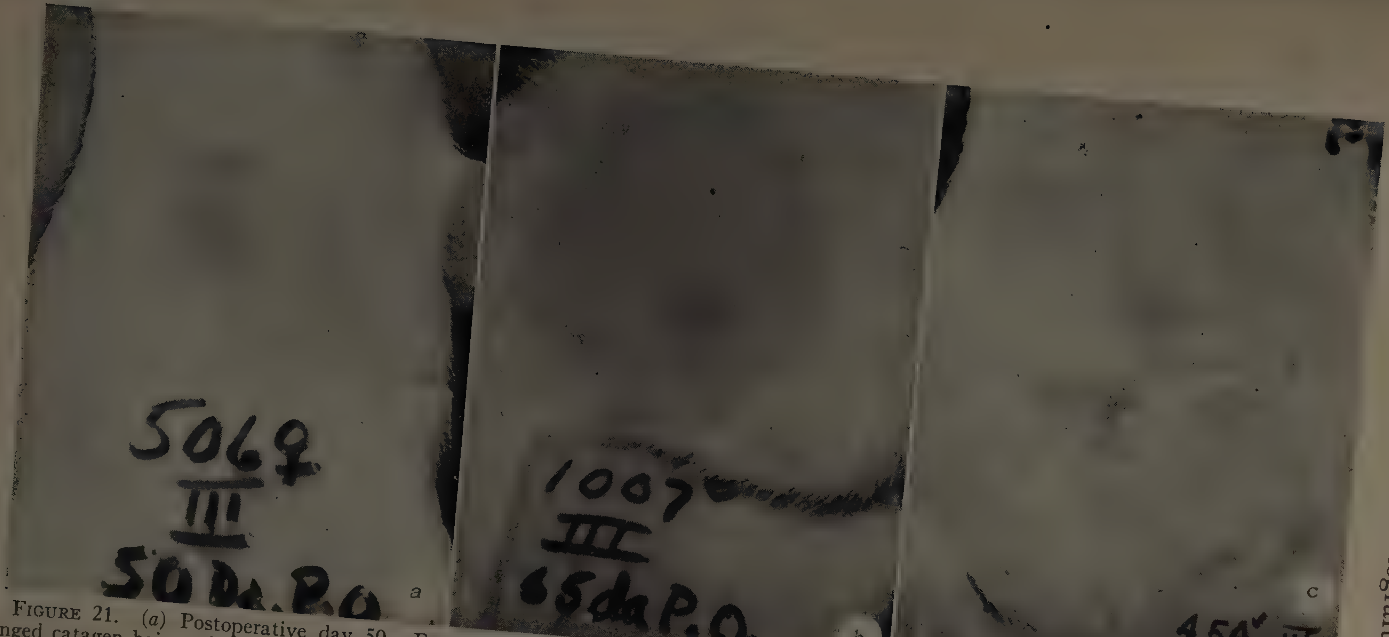

FIGURE 21. (*a*) Postoperative day 50. Female. Note that the hair is not actively growing as though the homograft were in a prolonged catagen hair cycle. For components of injection material *see* caption to FIGURE 5. (*b*) Postoperative day 65. Male. Exuberant hair growth and increase in the total area of the graft. (*c*) Postoperative day 93. Male. Almost complete obliteration of the line of surgical union of homograft with host.

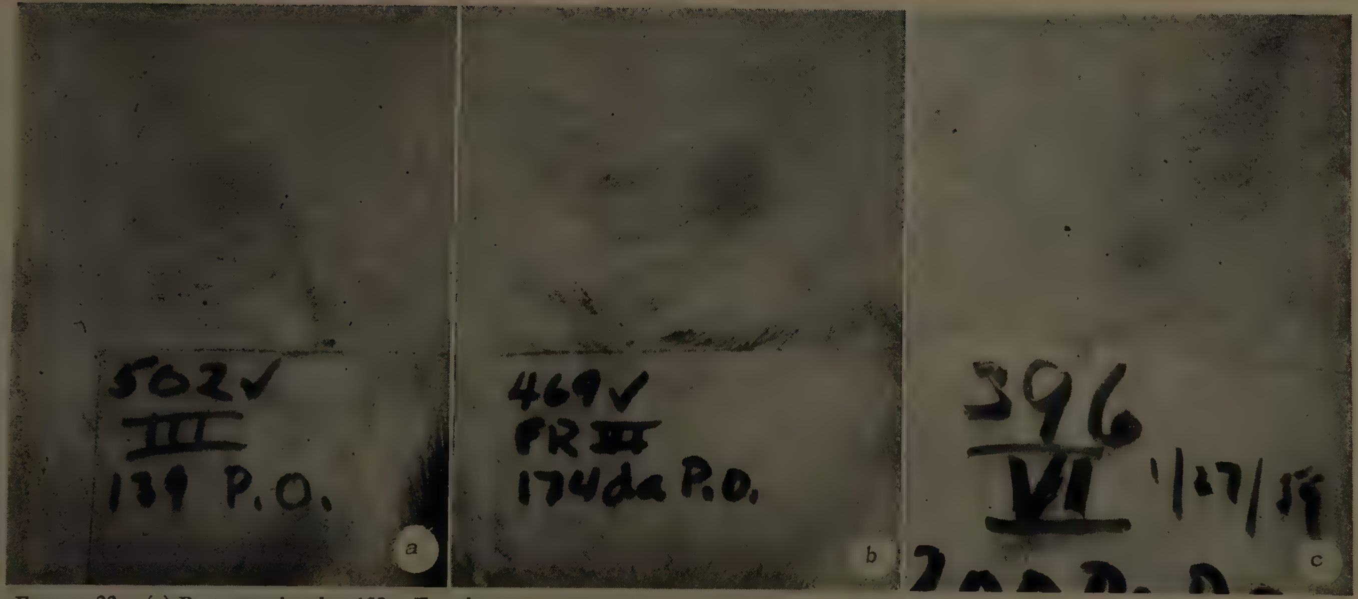

FIGURE 22. (*a*) Postoperative day 139. Female.
(*b*) Postoperative day 174. Male. The hair is coarser in texture than that seen in the female recipient in (*a*) or in the recipient host shown in (*c*).
(*c*) Postoperative day 200. Male. Approximately two thirds of the homograft is shown in this photograph.

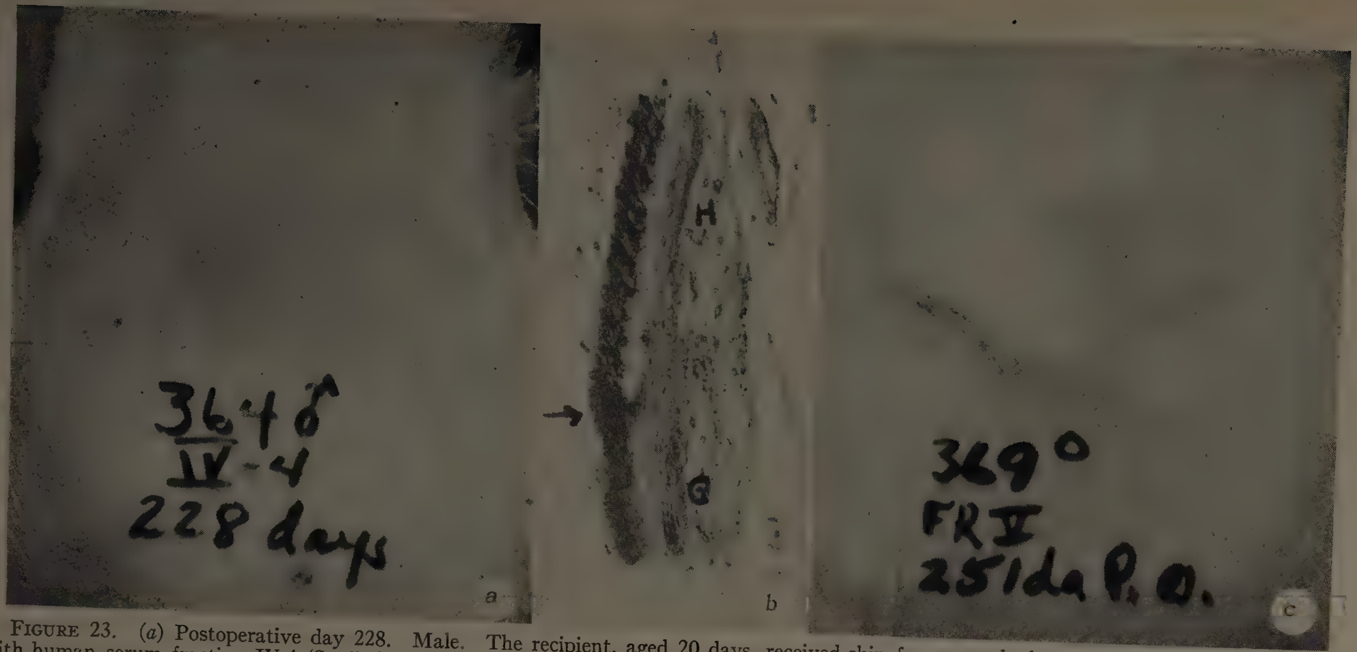

FIGURE 23. (*a*) Postoperative day 228. Male. The recipient, aged 20 days, received skin from a male donor aged 24 days and treatment with human serum fraction IV-4 (Squibb). Electrophoretic analysis showed this serum fraction to contain 59.5 per cent alpha$_1$ globulin, 33.9 per cent alpha$_2$ globulin, and 6.6 per cent beta globulin.

(*b*) Postoperative day 248. Female (×6). The corrugations are clearly seen on the homograft. The arrow points to the conjunctional line. There appears to be a greater development of the panniculus carnosus in the homograft area than in the host; the suprapannicular fat has not developed to any great extent in the homograft.

(*c*) Postoperative day 251. Male. Extensive coverage of host by enlarging homograft. Pooled rat serum fraction V consists of 47.6 per cent albumin, 27.0 per cent alpha$_1$ globulin, and 25.4 per cent alpha$_2$ globulin.

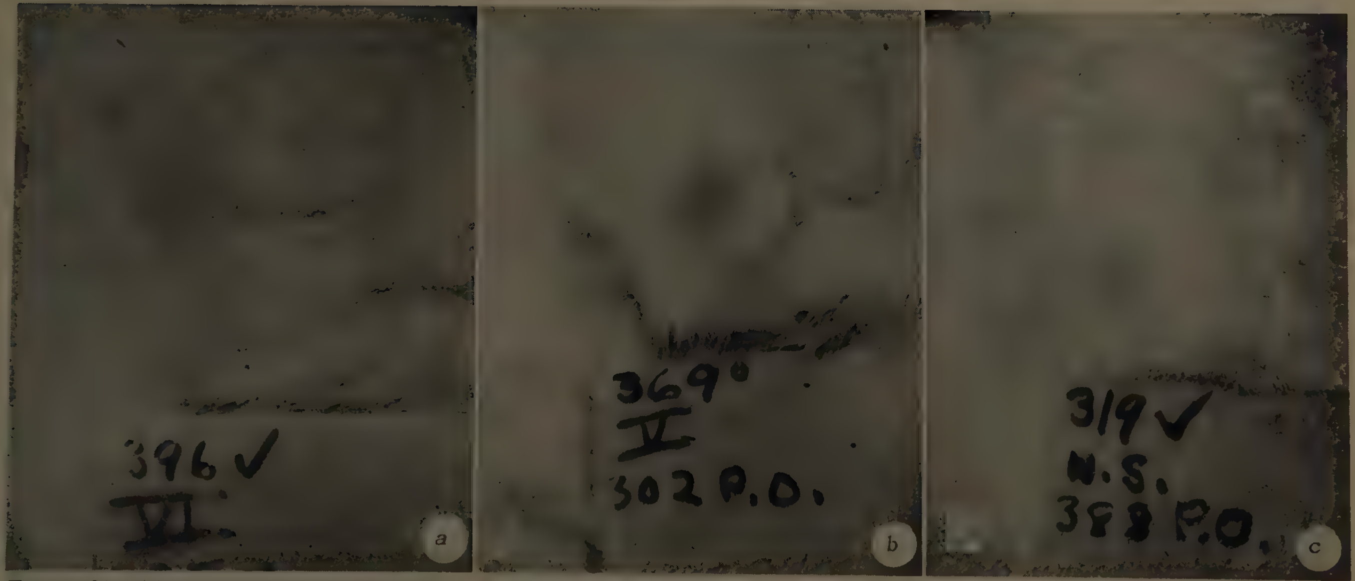

FIGURE 24. (*a*) Postoperative day 285. Male. Compare with FIGURE 22*c*.
(*b*) Postoperative day 302. Male. Compare with FIGURE 23*c*.
(*c*) Postoperative day 388. Male. The notation W.S. refers to lyophilized pooled whole rat serum. This animal was aged 23 days when skin from a male rat aged 21 days was transplanted on the dorsum. Although the recipient was clipped for the picture, the homograft shows sparse hair growth and has not increased greatly in surface area.

Discussion

Total growth and electrophoretic analysis. Statistical analysis of the means derived from the periodic weighing of the animals shows that standard deviation is about 2.0 per cent of the total weight at each age period. This finding suggests that the male control animal's weight is significantly greater than that of animals that have been subjected to unsuccessful skin grafting. The growth curves shown in FIGURE 1 also clearly indicate that an injected animal bearing a successful homograft is very similar in its weight increments to a normal untreated animal. There may be significance in the finding that the time when the successful homografted animals equal and exceed in weight that of the normal animals is the period when there is a depression in the initial high albumin titer. The work of Jeffay and Winzler (1958) suggests that such a close relationship of albumin turnover and nutrition exists.

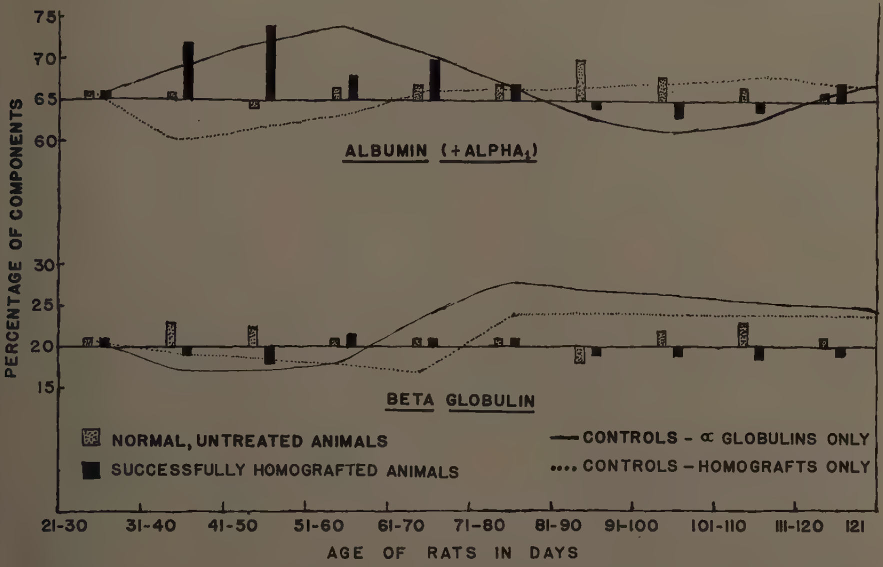

FIGURE 25. The bar graph shows changes in the serum percentage levels of albumin (plus alpha$_1$ globulin) and beta globulin in normal and successfully homografted animals. The line graph shows serum changes found only in animals injected with alpha globulin fractions (no homografts placed) and in untreated, unsuccessfully homografted animals. All serum analyses were made with Veronal buffer, *p*H 8.6; $\mu = 0.10$ in Antweiler microelectrophoresis apparatus.

Data of the weight relationships found in the female groups are difficult to explain. Only during the initial period of the first 40 days of life (approximately the fifteenth postoperative day) do the normal controls show a greater daily weight increment than do the experimental animals. In the case of animals bearing sloughing grafts this is understandable, as almost all animals of either sex show little gain or even loss of weight during the few days when the graft is being sloughed. The subsequent course during which the unsuccessfully homografted animals heal and increase greatly in weight would suggest that such active repair of a tissue loss acts as a stimulus. The female animals bearing successful grafts tend to parallel the normal animals, although at a higher level of nutritional intake. Since no studies were made of the nitrogen consumption and waste output, it is impossible to state whether there has been a change in the fluid and nutritional balances in these animals.

Homograft growth and histological changes. The tolerance of a homograft is a very interesting phenomenon. During days 5 or 6, when the dressings are removed, one finds that the probable successful homograft will have the same color as the host, and the clipped surface will appear cleaner, as though closely shaved. The sewed periphery of the graft margin will be merged with the tissues of the host so that a thin, slightly reddish line is evident. The graft will feel soft to the touch and freely movable over the underlying fascial plane.

Between postoperative days 7 and 12, a new crop of hair will be seen erupting from the surface of the graft. The junctional area of contact between the graft and host skin will show almost complete elimination of the suture line; this area also will be soft to the touch.

In males, from this time until postoperative day 28, there is a measurable decrease in size of the total surface area of the graft. In females this period of contraction is of greater degree and intensity, reaching its maximum by postoperative day 21. The diminution in the size of the graft is probably a product of (1) retraction of the host's panniculus carnosus, with subsequent replacement by peripherally disposed contractile connective tissue, (2) degenerative changes in the graft panniculus carnosus, with partial replacement by densely packed collagen fibers, (3) degenerative changes in the junctional areas of both host and graft, with subsequent condensation of connective tissue elements, and (4) the rapid loss of fatty tissue from the graft. The investigations of Van Den Brenk (1955, 1956) on the quantitative aspects of the contraction of skin wounds made in the flanks of rabbits (round excisions of 1.2 to 3.0 cm. diameter) can be compared to the present skin homograft study in rats. Van Den Brenk found that there was a latent period prior to contraction that averaged 9.4 days (±2.3). In discussing the effects of covering the wound with an autogenous graft, he suggests that contraction to a large extent is inhibited by "an equalization and stabilization of abnormal differential tissue tension created by injury. . . ." In conclusion, he states that "contraction is essentially a process of morphyllaxis (a general remodelling of the remaining portion of the body to restore the whole form), involving cellular migration and involves both epitheliocyte and mechanocyte tissues similar to morphyllactic phenomena which take place following injury in other tissues and organisms." From the results reported here it is quite evident that covering the wound with a skin homograft shortens the latent period and permits contraction to continue to a finite point, after which the homograft assumes the growth characteristics of the host.

Histological examination clearly demonstrates that epithelial union between host and graft occurs before there are any signs of formation of granulation. The curve for wound healing illustrated by Du Noüy (1936) suggests that epithelial regeneration and migration begin as an effort to reconstitute the loss of covering tissue when contraction ceases. This finding is not confirmed by the present work. Whether epithelization between host and graft is instrumental in bringing about contraction of the operated area is uncertain, as the migration and probable proliferation of cells appear to be a passive development. It is only after about 8 to 12 postoperative days have elapsed that one finds connective tissues lying parallel to the surface of the area of union and at right angles to the connective tissue elements in both host and graft. This finding would suggest that some internal tensions on the uniting tissues have occurred. At this period the epithelization is complete, however, and, in fact, it is in excess of the apparent requirements, as evidenced by the thickness of epithelial cells covering the host. This finding was also reported by Gillman *et al.* (1955–1956).

The presence of epithelial pegs from the heaped-up epithelial layer covering the junctional area suggests that these downgrowths presage formation of new hair follicles and sebaceous gland cells. Even when there is almost complete obliteration of the line of union between the 2 tissues the hyperepithelization persists in this area. To a great extent such obliteration is facilitated by the presence of hair follicles in the area, displacing or reorganizing the originally formed scar tissue. Breedis (1954), in his very interesting paper, points out that, when a wound in the skin was allowed to heal under conditions that prevented contraction and drying, numerous functioning hair follicles and sebaceous glands appear in the scar area. His experiments appear to demonstrate that these structures arose by budding from radially arranged bars of thickened epithelium, at least some of which had grown or migrated from cut hair follicles at the edge of the wound. Gillman *et al.* (1955–1956), suggests that such spurs as occur during the healing of skin wounds in humans are later separated from the surface parent epithelium and form embedded keratinizing epithelial pearls. On the other hand, Durward and Rudall (1949) do not mention this finding, despite extensive histological examination of skin autografts in rats.

The precise sequence of events that occurs in the panniculus carnosus during homografting is not clear. The present investigations suggest that, soon after the skin transplant is placed, there is a retraction of the cut fibers and an apparent swelling of the fasciculi. By the fourth day there is an invasion of the muscle fibers by capillaries and fibroblasts, with a varying amount of histiocytes. The fibers lose their cross striations and appear disrupted. With complete tolerance of the graft the muscle bundles appear to diminish in size and number while being enclosed by a firm, fibrous sheath. From postoperative day 15 there appears to be a return of cross striations to the remaining muscle fibers. The previously fragmented and, in some instances, amorphous structure is replaced by normal-appearing muscle fibers. In some cases, however, by postoperative day 35 many of the muscle fibers making up the graft panniculus carnosus have disappeared; in others they appear to have increased in size and number.

In a very excellent study, Clark (1946) showed that autografts of rabbit gracilis muscle completely degenerated by day 3. His histological sections of the tissue showed that by day 11 there were signs of regeneration of the muscle in the autograft, as indicated by staining reactions and the presence of cross striations. However, after careful investigation Clark concluded that he had no evidence that regenerating muscle arises by any means other than a continuous outgrowth from the stumps of old, pre-existing fibers, that is, from the undamaged muscle of the host.

Conclusions

The study of the various events that occur in the acquired tolerance of albino rats to skin homografts shows that:

(1) The injection of serum fractions containing alpha globulins concomitant with skin homografting conditions the recipient so that it maintains its homeostatic state. This belief is based upon the similarity of the serum components in normal and successfully homografted animals when healing is complete; that is, about 80 days of age or about postoperative day 55. It is also supported by the evidence that the animals maintain their weight in a manner comparable to that of normal, untreated animals.

(2) Acceptance of a skin homograft occurs most frequently when exchanged between animals of the same sex. Its subsequent growth curve is dependent upon the sex of the recipient.

(3) Histological data support recent workers who have reported that epithelial migration and regeneration from the wound edges of the host commence within the first 24 hours. By day 4 the junctional area between host and graft has been covered completely.

(4) Downgrowth of epithelial spurs from the hyperepithelized covering of the junctional area and the subsequent appearance of new hair follicles and sebaceous glands in the area strongly suggest that these structures are newly formed.

(5) The final course of events that affect the denervated panniculus carnosus is uncertain. The data make it appear that some of the muscle fibers persist and re-establish themselves, but such muscle fibers may have been an ingrowth from the uninjured host's panniculus carnosus.

References

Antweiler, H. J. 1952. Die Quantitative Elektrophorese in der Medizin. Springer-Verlag. Berlin, Germany.

Arey, L. B. 1936. Wound healing. Physiol. Rev. **16:** 327.

Ballantyne, D. L., Jr. & J. M. Converse. 1957. The relation of hair cycles to the survival time of suprapannicular and subpannicular skin homografts in rats. Ann. N. Y. Acad. Sci. **64**(5): 958.

Ballantyne, D. L., Jr. & J. M. Converse. 1959. Further observations of hair cycles and the survival of skin homografts in rats. Transplantation Bull. **6:** 93.

Breedis, C. 1954. Regeneration of hair follicles and sebaceous glands from the epithelium of scars in the rabbit. Cancer Research. **14:** 575.

Carrel, A. & P. L. Du Noüy. 1921. Cicatrization of wounds. XI. Latent periods. J. Exptl. Med. **34:** 339.

Clark, W. E. LeG. 1946. An experimental study of the regeneration of mammalian striped muscle. J. Anat. **80:** 24.

Du Noüy, P. L. 1936. Biological Time. Methuen. London, England.

Durward, A. & K. M. Rudall. 1949. Studies on hair growth in the rat. J. Anat. **83:** 325.

Edwards, L. C. & J. E. Dunphy. 1958. Wound healing. I. Injury and normal repair. II. Injury and abnormal repair. New Engl. J. Med. **259:** 224; 275.

Eichwald, E. J., C. R. Silmser & N. Wheeler. 1957. The genetics of skin grafting. Ann. N. Y. Acad. Sci. **64**(5): 737.

Gillman, T., J. Penn, D. Bronks & M. Roux. 1955–1956. A re-examination of certain aspects of the histogenesis of the healing of cutaneous wounds. Brit. J. Surg. **43:** 141.

GJESSING, E. C. & A. CHANUTIN. 1947. An electrophoretic study of plasma and plasma fractions of normal and injured rats. J. Biol. Chem. **169:** 657.

HARDIN, C. A. & A. A. WERDER. 1955. A one year study of surviving homografted mouse skin. Plastic and Reconstruction Surg. **15:** 107.

HUGHES, A. F. W. & L. DANN. 1941. Vascular regeneration in experimental wounds and burns. Brit. J. Exptl. Pathol. **22:** 9.

JEFFAY, H. & R. J. WINZLER. 1958. The metabolism of serum proteins. I. The turnover rate of serum proteins. II. The effect of dietary protein on the turnover of rat serum proteins. J. Biol. Chem. **231:** 101; 111.

KAMRIN, B. B. 1958*a*. Reciprocal blood exchange in parabiosed albino rats. Am. J. Physiol. **194:** 507.

KAMRIN, B. B. 1958*b*. The use of globulins as a means of inducing acquired tolerance to parabiotic union. Ann. N. Y. Acad. Sci. **73**(3): 848.

KAMRIN, B. B. 1959. Successful skin homografts in mature non-littermate rats treated with fractions containing alpha-globulins. Proc. Soc. Exptl. Biol. Med. **100:** 58.

LINDQUIST, G. 1946. The healing of skin defects. Acta Chir. Scand. **94** (Suppl. 107.): 1.

MARCHAND, P. J. 1901. Der Process Wundheilung. (Deutsche Chirugie, Lief 16) 528 pp. Enke. Stuttgart, Germany.

MEDAWAR, P. B. 1954. General problems of immunity. *In* Preservation and Transplantation of Normal Tissues. : 1–20. Little, Brown. Boston, Mass.

MEDAWAR, P. B. & M. F. A. WOODRUFF. 1958. The induction of tolerance by skin homografts on newborn rats. Immunology. **1:** 27.

NEEDHAM, A. E. 1952. Regeneration and Wound Healing. 152 pp. Methuen. London, England.

RANDALL, P. & I. M. DUSHOFF. 1958. Skin cycles and other physiological variables of rodent skin: The effect of the skin cycle on skin homograft survival in the mouse. Plastic and Reconstruction Surg. **21:** 24.

ROTH, S. 1954. Incorporation of N^{15} into protein and P^{32} into nucleic acid of the thermally injured rat. Am. J. Physiol. **176:** 471.

SCHULTZ, J. 1959. Antigens and antibodies as cell phenotypes. Science. **129:** 937.

SNELL, G. D., E. RUSSELL, E. FEKETE & P. SMITH. 1953. Resistance of various inbred strains of mice to tumor transplants and its relation to the H-2 locus which each carries. J. Natl. Cancer Inst. **14:** 485.

TOOLAN, H. W. 1954. Growth of human embryonic tissues in cortisone treated laboratory animals. Proc. Soc. Exptl. Biol. Med. **86:** 607.

TOOLAN, H. W. 1958. Studies of adult and embryonic skin homografts on conditioned or normal rabbits, with emphasis on the possible role of the ground substance. Ann. N. Y. Acad. Sci. **73**(3): 546.

TRENTIN, J. J. 1958. Tolerance and homologous disease in irradiated mice protected with homologous bone marrow. Ann. N. Y. Acad. Sci. **73**(3): 799.

VAN DEN BRENK, H. A. S. 1955–1956. Studies in restorative growth processes in mammalian wound healing. Brit. J. Surg. **43:** 525.

WILLIAMSON, M. B. (Ed.) 1957. The Healing of Wounds: A Symposium of Recent Trends and Studies. 213 pp. McGraw-Hill. New York, N. Y.

WOODRUFF, M. F. A. & L. O. SIMPSON. 1955. Experimental skin grafting in rats: With special reference to split skin grafts. Plastic and Reconstruction Surg. **15:** 451.

THE ACTION OF ANTIBODY AND COMPLEMENT ON MAMMALIAN CELLS*

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The studies discussed in this paper are concerned principally with the action of heterologous antibodies on mammalian cells. Cell-antibody interactions are most conveniently studied in such systems, for it is a simple matter to obtain powerful heterologous antisera. However, the action of homologous antibodies now has been studied [1-4] (*see also*, C. A. Stetson and E. Jensen, this monograph) sufficiently to make it clear that they produce the same kind of changes in cells as do heterologous antibodies. There is therefore no reason to doubt that the mechanism of action on cells is substantially the same for the two kinds of antibodies.

The effects of antibodies will be described as they are observed *in vitro* under conditions in which it is possible to distinguish between the effects of antibody alone and those requiring the presence of complement. How these effects may be modified *in vivo* when the cells are in the form of a solid tissue transplant will not be considered in this paper.

Most studies of the *in vitro* effects of antibodies on animal cells have utilized erythrocytes because they represent a homogeneous population of cells uniformly exposed to the action of substances in the medium. Methods of obtaining tissue cells in free suspension or in monolayers have provided similar advantages in the use of structurally more complex cells than the anucleate erythrocyte. Although the events occurring in such cells when they are exposed to antibody and complement differ in detail from those in the erythrocyte, the mechanism of immune lysis in the two cases appears to be the same.

The Effects of Antibody on Animal Cells in the Absence of Complement

When cells in free suspension are treated *in vitro* with specific antibodies and studied by phase or light microscopy, agglutination appears to be the major morphologic change.[3,5] An electron microscopic study on the Krebs mouse ascites tumor cell[5] has demonstrated that the nucleus, mitochondria, lipid inclusions, the endoplasmic reticulum and ribosomes were unaffected by antibody action. The cell membrane, however, was distinctively altered by exposure to the antitumor cell gamma globulin. The alteration took the form of a focal series of projections and invaginations of the surface membrane with the corresponding production of narrow channels extending into the cytoplasmic matrix. The altered surfaces of apposed cells frequently interdigitated, indicating that the transformation favored agglutination.

It seems possible that this folding of the cell surface could have been brought about by normal surface membrane movements (pinocytosis?) bringing separated antigenic points in the cell membrane into sufficiently close proximity to

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allow bridging by divalent antibody. Other antibody molecules could then act in "zipper fashion" to fix apposed surfaces. If membrane movements persisted in immediately adjacent zones, the process could extend locally.

Another kind of structural evidence for antigen-antibody binding in cellular membranes has been provided by the electron-microscopic studies of Bessis[6] and Rebuck[7,8] on erythrocytes exposed to specific blood group antibodies.

The available evidence supports the view that antibody alone does not affect the selective permeability or chemical constitution of cells[9] or their metabolic properties.[10,11] Indeed, mammalian cells can be grown in the presence of significant concentrations of antibody.[12-14] Goldstein and Myrvik[12] have propagated HeLa cells over 15 subcultures in antibody concentrations 5 to 10 times higher than were necessary to destroy 90 per cent of the cells upon the addition of complement. The growth rate, however, was much below that of the control cultures, and clumping, microcolony formation and some changes in cell shape were noted. Quersin-Thiry[13] has grown HeLa cells at normal rates in antibody concentrations insufficient to cause agglutination, but still capable of protecting the cells against the attachment of polio virus. There is, therefore, considerable evidence that, although antibody combines with antigenic sites in the cell membrane and may impose alterations of surface structure, it does not seriously affect the viability or metabolic functions of the cell.

The Effects of Antibody and Complement on Animal Cells

Morphologic changes. In contrast to the relatively innocuous effects of antibody alone, the combined actions of antibody and complement are highly lethal to mammalian cells. Morphologically, a variety of cytotoxic changes are noted (*see* review by Wissler and Flax,[15] also Goldberg and Green,[5] Goldstein and Myrvik,[12] Lumsden,[14] Latta and Kutsakis,[16] and Bickis *et al.*[11]). Although the details of the structural damage produced may have been modified by the particular *in vivo* or *in vitro* conditions employed, most of the studies have indicated that the affected cells become swollen by the entrance of water. With cells in monolayers or in free suspension, the process characteristically begins by the formation of numerous peripheral blebs,[5,11,16,17] but may finally result in a more or less uniformly swollen cell. Electron microscopic studies of cells in tissue culture[18] and in free suspension[5] have confirmed the finding that there is a movement of fluid into membrane-bounded compartments, including mitochondria, endoplasmic reticulum, and perinuclear space.

At least in the case of antibody-complement treated cells in free suspension, the cell membrane remains unbroken during these events,[1,5] but the cells become very fragile and can be disrupted easily by mechanical forces.[19]

Chemical changes. The earliest studies were performed on Ehrlich ascites tumor cells by Colter *et al.*[20] and Ellem,[21,22] and the results were similar to those obtained on Krebs ascites tumor cells.[9,23]

The earliest and most striking change was the rapid equilibration of small molecules between the cell interior and the medium. K^+ was lost from the cells at a rate far greater than its normal leak rate and Na^+ passed rapidly into the cells. The cells quickly lost the bulk of their phosphates,[22] amino acids,[23] and ribonucleotides.[21,23] The macromolecules of the cytoplasm—RNA and protein—were also lost,[9,20,23] but at a slower rate, while desoxyribonucleic acid remained in the cells.[9,20] The chemical data are consistent with the electron microscopic observation that large numbers of 150A ribonucleoprotein particles (ribosomes) had disappeared from the cytoplasmic matrix of antibody-complement-treated Krebs ascites cells,[5] and the bulk of the ribonucleic acid lost from the cells could be recovered as intact ribosomes by centrifugation (105,000 g) of the cell-free medium.[19] Larger structures, however, do not pass into the medium. Thus, in immune cytolysis as in immune hemolysis, leakage of intracellular macromolecules occurs through a stretched but morphologically intact cell membrane.

Metabolic changes. In view of the large losses of cell constituents from antibody-complement-treated cells, it is not surprising that metabolic properties of the cells are seriously affected. Substrates, coenzymes, and enzymes are probably lost, although perhaps to varying degrees, depending on the molecular sizes of the substances and their locations in the cell. This could explain the varying susceptibility of different metabolic processes to the action of antibody and complement. For example, the incorporation of C^{14} glycine into cell protein is prevented nearly completely by antibody and complement,[11,20] and anerobic glycolysis,[11] endogenous respiration,[11] and oxygen consumption in the presence of glucose[10,11] are abolished. On the other hand, treated cells continue to metabolize succinate, even at an increased rate.[10,11] This could be due to retention within the cell of the particulate succinic dehydrogenase system of the mitochondria as opposed to the loss of the soluble glycolytic enzymes of the cytoplasm.

Mechanism of the Permeability Alteration Produced by Antibody and Complement

The mechanism of production of the observed changes in antibody-complement-treated cells is believed to be the following: the normal cell contains a considerably higher protein concentration than the extracellular fluid, but there is normally no osmotic pressure gradient across the cell membrane. This is thought to be accomplished by the action of energy-dependent pumps which, by maintaining the ion concentration inside the cell slightly lower than that outside, compensate for the colloid osmotic pressure gradient. Any condition that leads to equilibration of ions between cell and medium therefore would produce an unbalanced colloid osmotic pressure gradient across the cell membrane, and water would flow into the cell. Such a sequence of events has been produced by various means in erythrocytes, where it has been called colloid osmotic hemolysis.[24]

It was first suggested by Ellem[22] that immune lysis might be a form of colloid osmotic lysis, and recent studies[23] have shown that this is indeed the case. Antibody and complement do cause the equilibration of ions between cell and medium and an osmotic shift of water into the cell. However, the swelling can be prevented in spite of the ionic equilibration by adding bovine serum albumin (BSA) to the medium in a concentration sufficient to balance the colloid osmotic pressure of the cell[23] (H. Green and F. Silverblatt, unpublished observations). Under these conditions, macromolecules (protein and RNA) are not lost from the tumor cells. In antibody-complement-treated erythrocytes the same holds true: K^+ is lost from the cells, but hemoglobin is retained when albumin is present in the medium in sufficient concentration.[23]

Therefore, it would appear that the direct action of antibody and complement is to produce "holes" in the cell membrane through which small molecules equilibrate, but osmotic swelling of the cell and stretching of the cell membrane are required for the escape of macromolecules.

Comparative Studies of Osmotic Swelling

If Krebs ascites tumor cells are induced to swell by a variety of means, it can be shown that the loss of macromolecules is not exclusively a function of the degree of swelling. For example, when the cells were incubated at 0° C., the metabolically dependent pumps failed to maintain the normal cation gradients. After a short interval, therefore, cation equilibration occurred, and colloid osmotic swelling resulted. After about 6½ hours, the cells were swollen to almost 3 times normal size, but there was no loss of protein or RNA (TABLE 1, EXPERIMENT 1).

TABLE 1

A COMPARATIVE STUDY OF OSMOTIC SWELLING IN ASCITES TUMOR CELLS

	Experiment 1 Cold swelling, 6.5 hrs., 0° C.			Experiment 2 Hypotonic swelling, 20 min., 37° C.			Experiment 3 Immune swelling, 20 min., 37° C.		
	Control	Expt'l.	Expt'l./Control	Control	Expt'l.	Expt'l./Control	Control	Expt'l.	Expt'l./Control
Packed cell volume (mm.^{3})	10.5	30.0	2.86	8.05	18.5	2.30	11.7	27.6	2.36
Cell protein (mg.)	4.25	4.09	0.96	4.32	4.41	1.02	4.72	3.26	0.69
Cell RNA (mg.)	0.71	0.72	1.01	0.74	0.71	0.96	0.70	0.34	0.48

Methods of preparation of the cells and incubation conditions have been described previously.[5] BSA was added in EXPERIMENTS 1 and 2 to a final concentration of 0.1 per cent to prevent sticking of the cells to the tube wall during centrifugation. Packed cell volumes were determined with capillary sedimentation tubes.[25] Protein was determined by the method of Lowry *et al.*[26] RNA was separated by the method of Schmidt and Thannhauser[27] and quantitatively measured by the orcinol method of Dische.[28]

Tumor cells were also swollen by the slow addition of distilled water to a suspension of the cells in isotonic balanced salt solution (TABLE 1, EXPERIMENT 2). Here too, swelling of the cells to 2.3 times normal volume was not accompanied by loss of RNA or protein. However, further reduction in the salt concentration of the medium did induce a greater degree of swelling and appreciable loss of macromolecules without bursting of the cells (see also Ellem[21]).

Included in TABLE 1 for comparison is an experiment in which cell swelling was induced by the action of antibody and complement (EXPERIMENT 3). For a degree of swelling comparable to that of the other experiments, there was abundant loss of both protein and RNA. Therefore, for comparable degrees of stretching, the membrane of an antibody-complement-treated cell was more permeable to macromolecules than a normal cell membrane.

The concept that antibody and complement produce "holes" in the cell membrane is useful for the interpretation of differences between immune lysis and other forms of osmotic lysis. Bordet was able to show many years ago[29] that erythrocyte ghosts produced by distilled water lysis could be shrunk by the addition of hypertonic sodium chloride, while those produced by immune lysis could not. Evidently, after release of their hemoglobin, distilled water-lysed erythrocytes again become relatively impermeable to small molecules, while immune-lysed cells remain permeable.

A distinction also may be made with respect to erythrocytes lysed by a variety of other agents and immune lysed erythrocytes. Erythrocytes acted on by *Clostridium septicum* toxin,[30] resorcinol,[31] salicylate, X ray and ultraviolet irradiation, photodynamic action and other agents[24] undergo colloid osmotic hemolysis, but the cells may be protected from lysis by sucrose, which is able, as a nonpenetrating solute, to balance the colloid osmotic pressure of hemoglobin and prevent swelling. However, erythrocytes acted upon by antibody and complement are not appreciably protected from hemolysis by sucrose, because sucrose is able to enter the cells rapidly through the holes made by antibody and complement. In this case, in order to prevent lysis of the cells, it is necessary to use a large molecule such as albumin, which cannot penetrate the altered membrane.

Once lesions are produced by antibody and complement in the cell membrane and cation equilibration has occurred, lysis is probably inevitable if the colloid osmotic pressure of the cytoplasm is not balanced by macromolecules in the medium. However, the time required for equilibration and lysis to occur would probably depend upon the number of holes made in the surface membrane. Under conditions where the number of lesions is very small, the time required for the loss of macromolecules might be considerable.

The Effects of Molecules of Different Size in Preventing Colloid Osmotic Lysis of Antibody-Complement-Treated Cells

Colloid osmotic lysis of antibody-complement-treated cells can be prevented by the addition of a substance to the medium only if its molecular size and configuration prevent its passage through the altered membrane. By the addition of substances of different molecular weights, a rough estimate could therefore be made of the size of the holes as they are made by antibody and complement. Substances suitable for such a study must have the following properties: (1) they must be very soluble in water; (2) they must be nontoxic to the cell (basic polymers, for example, interact strongly with cells and are very toxic); and (3) they must neither inactivate antibody or complement nor interfere with their action on the cell membrane.

The polyvinyl pyrollidones (PVP) satisfied these criteria, at least for the most part. However, they fell short of the ideal in several respects: (1) at high concentrations, they precipitated small quantities of protein from the gamma-globulin and complement solutions; and (2) they were not homogenous products with respect to molecular size, and even their average molecular weights had been determined only approximately. The PVP preparations used in these experiments, K-30 and K-15,* were characterized by viscosity

* General Aniline and Film Corp., New York, N. Y.

measurements. An approximate empirical relationship has been established between the Fikentscher values obtained from viscosity measurements and molecular weights; in this way, mean molecular weights of 40,000 and 13,000 have been assigned to K-30 and K-15, respectively, by their manufacturer.

A comparison was made between PVP, BSA, and sucrose as inhibitors of immune hemolysis. FIGURE 1 shows the results of an experiment in which

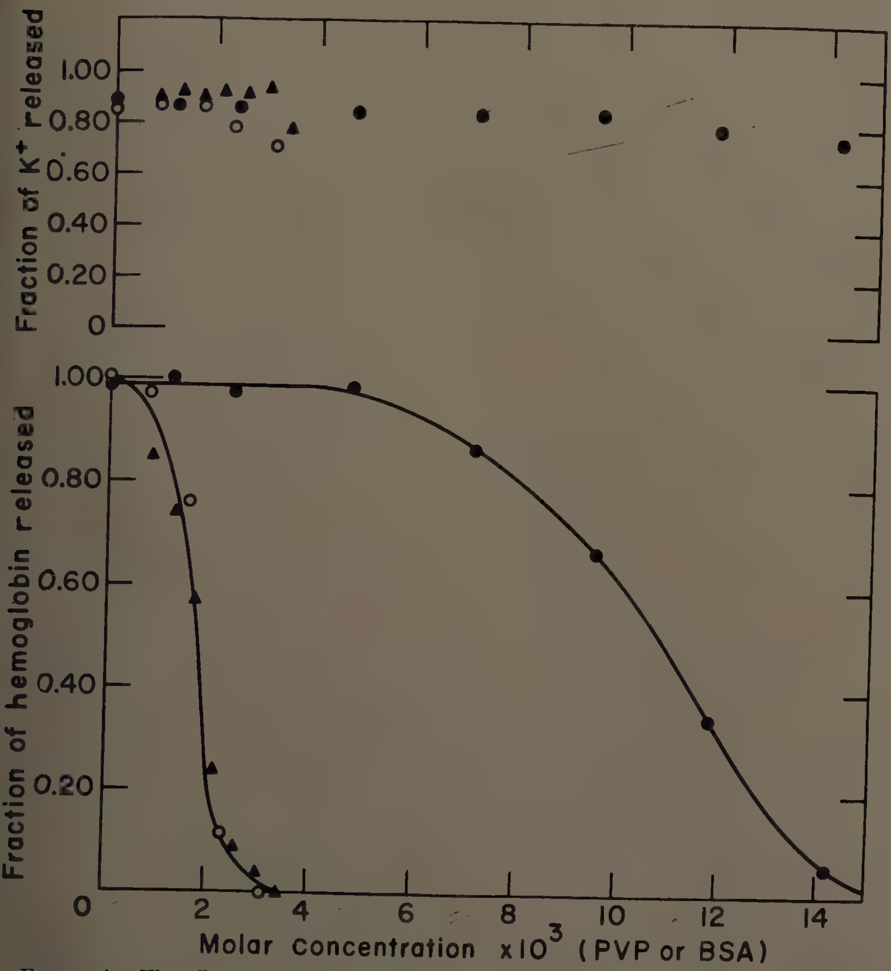

FIGURE 1. The effect of PVP and BSA on the release of K^+ and hemoglobin in immune emolysis. Into a series of tubes were placed 1.0 ml. of different concentrations of PVP or SA, 0.050 ml. of rabbit-immune gamma globulin (66 mg./ml.), 0.050 ml. of washed 50 per ent suspension of mouse erythrocytes, and either 0.20 ml. of complement (fresh rabbit serum) r 0.20 ml. of heat-inactivated complement. After 15 min. incubation at 37° C., the suspenons were centrifuged and the hemoglobin concentrations of the supernatants determined y their optical density at 541 mμ, using the corresponding inactivated complement supernaant as the blank. The cell pellets were analyzed for K^+ by flame photometry. The results re expressed as fractions of the total hemoglobin or K^+ of the cells released to the medium. ey: ▲ = BSA; ○ = PVP, K-30; ● = PVP, K-15.

mouse erythrocytes were incubated in solutions of these substances at various concentrations. Rabbit-immune gamma globulin and complement were added and, after incubation, the amount of hemolysis was determined. It may be seen that BSA (molecular weight, 65,000) and PVP (K-30) were approximately of equal effectiveness, on a molar basis, in protecting the erythrocytes against immune hemolysis. At concentrations of 1.8×10^{-3} M hemolysis was 50 per cent inhibited and, at 3.0×10^{-3} M, completely inhibited. PVP (K-15) was very much less effective in protecting the erythrocytes, a sixfold higher molar concentration being necessary to inhibit hemolysis by 50 per cent. Sucrose was ineffective at concentrations up to 0.5 M.

It will be noted that there was virtually complete release of K^+ from the antibody-complement-treated cells at all the PVP and BSA concentrations shown. It is therefore clear that the protection against hemolysis afforded by BSA and PVP was not due to inhibition of the action of complement or antibody. However, at concentrations higher than those shown here, both PVP and BSA can diminish the loss of K^+ as well. This might be due to interference with the action of antibody and complement, although other explanations are also possible.

A similar study of the ability of PVP, BSA, and sucrose to inhibit the swelling of antibody-complement-treated Krebs ascites tumor cells was based on comparisons of packed cell volumes after incubation of the suspensions. The results showed a very similar pattern over the same concentration ranges shown in FIGURE 1. PVP (K-30) and BSA were equally effective, PVP (K-15) considerably less effective, and sucrose virtually ineffective (F. Silverblatt and H. Green, unpublished observations).

It appears from these data that the holes made in the cell membrane by the action of antibody and complement were sufficiently large to admit a significant part of the low molecular-weight PVP preparation, but not substances of molecular weight range 40,000 to 65,000. Precise determination of the hole size was prevented by the fact that the PVP preparations were not homogeneous, and also by the short time (15 min.) allotted for equilibration. As the fit between hole size and molecule size becomes closer, the time required for equilibration would become longer.

Location of the Antigens Responsible for Immune Cytotoxicity

The occurrence of indifferent antigen-antibody reactions in close proximity to, or even perhaps within the ascites tumor cells, apparently has no effect on the cells. Tumor cells were incubated in the presence of various proteins for intervals of several hours, and the suspensions were then injected into mice immunized by a series of injections of the respective proteins in Freund's adjuvant. Some of the combinations used are shown in TABLE 2. Incubations were also performed in the presence of heat-denatured proteins, for it has been shown that such proteins are taken up by the cells at greatly increased rates (H. Green, unpublished observations). In all cases, the cells grew out as rapidly in mice immunized against the specific proteins as cells incubated for the same interval in the absence of the protein and injected into nonimmune mice. Also, incubation of the cells first in antigen, then in rabbit antiserum to the antigen, did not delay the growth of the tumor cells when they were subsequently injected into mice.

These results are not surprising, for in all probability only the combination of certain cellular antigens with their respective antibodies and complement results in permeability changes. Since all the results discussed earlier point to the cell membrane as the site of action of antibody and complement, it would seem reasonable that membrane antigens are responsible. The results of studies on the cytotoxic effects of antibodies produced by immunization with cell fractions are probably not incompatible with this view. Antibodies prepared in rabbits against soluble proteins of mouse ascites tumor cells were shown by Colter *et al.* to be ineffective in damaging the tumor cells as judged by their failure to inhibit cell growth[32] or to produce loss of cell protein and RNA.[20] Antibodies produced against ribonucleoprotein and desoxyribonucleoprotein fractions, however, were effective but one wonders if the preparations did not contain membrane antigens.

TABLE 2

Cells incubated in	Protein conc. (mg./ml.)	Incubation time (min.)
Human serum albumin	0.085	180
Heated (80° C., 60 min.) human serum albumin	0.085	180
Limulus hemocyanin	1.0	225
Sulfanilic acid azo hemocyanin	0.10	225
Bovine gamma globulin	1.0	225
Sulfanilic acid azo bovine gamma globulin	1.0	225
Sulfanilic acid azo human fibrinogen	1.0	225

Conclusions

Recent studies on the action of antibodies and complement on mammalian cells *in vitro* permit the following conclusions:

Specific antibodies may alter the ultrastructure of the cell membrane, but the permeability properties and metabolism of the cells remain unaffected. Mammalian cells can remain viable in the presence of appreciable concentrations of antibody.

Antibody and complement are both required to produce irreversible cell damage, and the cell membrane appears to be the site of their action. A leakiness in the cell membrane is induced that permits the loss of low molecular weight constituents and the equilibration of cations between the cell and the surrounding medium. The equilibration leads inevitably to the entrance of water into the cell along a colloid osmotic pressure gradient. The cell is expanded, the cell membrane stretched, and "holes" of sufficient size are made in the membrane to permit the escape of macromolecules into the medium.

These events represent a form of osmotic lysis, but immune lysis differs from osmotic lysis induced in other ways in the greater degree of leakiness produced in the cell membrane. The cell membrane exposed to antibody and complement is readily permeable to molecules of the size of sucrose and larger and, for comparable degrees of osmotic stretching, only the immune damaged membrane allows macromolecules to escape from the cell.

References

1. Kalfayan, B. & J. G. Kidd. 1953. Structural changes produced in Brown-Pearce carcinoma cells by means of a specific antibody and complement. J. Exptl. Med. **97:** 145–163.
2. Gorer, P. A. & P. O'Gorman. 1956. The cytotoxic activity of isoantibodies in mice. Transplantation Bull. **3:** 142–143.
3. Schrek, R. & F. W. Preston. 1956. Toxicity of homologous immune serum to a transplantable tumor: studies using phase microscopy and cinemicrography. J. Natl. Cancer Inst. **16:** 1021–1045.
4. Terasaki, P. I., J. A. Cannon & W. P. Longmire. 1959. Antibody response to homografts. Proc. Soc. Exptl. Biol. Med. **102:** 280–284.
5. Goldberg, B. & H. Green. 1959. The cytotoxic action of immune gamma globulin and complement on Krebs ascites tumor cells. I. Ultrastructural studies. J. Exptl. Med. **109:** 505–510.
6. Bessis, M. 1950. Studies in electron microscopy of blood cells. Blood. **5:** 1083–1098.
7. Rebuck, J. W. 1953. Structural changes in sensitized human erythrocytes observed with the electron microscope. Anat. Record. **115:** 591–613.
8. Rebuck, J. W. 1959. *In* Mechanisms of Hypersensitivity. Henry Ford Hospital, Intern. Symposium. : 138–141. Little, Brown. Boston, Mass.
9. Green, H., R. A. Fleischer, P. Barrow & B. Goldberg. 1959. The cytotoxic action of immune gamma globulin and complement on Krebs ascites tumor cells. II. Chemical studies. J. Exptl. Med. **109:** 511–521.
10. Flax, M. H. 1956. The action of anti-Ehrlich ascites tumor antibody. Cancer Research. **16:** 774–783.
11. Bickis, I. J., J. H. Quastel & S. I. Vas. 1959. Effects of Ehrlich ascites antisera on the biochemical activities of Ehrlich ascites carcinoma cells *in vitro*. Cancer Research. **19:** 602–607.
12. Goldstein, G. & Q. N. Myrvik. 1958. The reversible and irreversible toxic effects of anti-HeLa cell rabbit serum upon human cell lines in tissue culture. J. Immunol. **80:** 100–105.
13. Quersin-Thiry, L. 1958. Action of anticellular sera on virus infections. J. Immunol. **81:** 253–260.
14. Lumsden, C. E. 1959. Effects of antibodies on cells in tissue culture. *In* Immunopathology, 1st Intern. Symposium. : 262–277. P. Grabar and P. Miescher, Eds. Benno Schwabe. Basel, Switzerland.
15. Wissler, R. W. & M. H. Flax. 1957. Cytotoxic effects of antitumor serum. Ann. N. Y. Acad. Sci. **69**(4): 773–794.
16. Latta, H. & A. Kutsakis. 1957. Cytotoxic effects of specific antiserum and 17-hydroxycorticosterone on cells in tissue culture. Lab. Inv. **6:** 12–27.
17. Miller, D. G. & T. C. Hsu. 1956. The action of cytotoxic antisera on the HeLa strain of human carcinoma. Cancer Research. **16:** 306–312.
18. Latta, H. 1959. A cellular reaction to antibody in tissue culture studied with electron microscopy. J. Biophys. Biochem. Cytol. **5:** 405–410.
19. Goldberg, B. & H. Green. Immune cytolysis. J. Biophys. Biochem. Cytol. In press.
20. Colter, J. S., D. Kritchevsky, H. H. Bird & R. F. J. McCandless. 1957. *In vitro* studies with antisera against tumor cell protein fractions. Cancer Research. **17:** 272–276.
21. Ellem, K. A. O. 1957. Studies on the mechanism of the cytotoxic action of antisera. Australian J. Sci. **20:** 116–117.
22. Ellem, K. A. O. 1958. Some aspects of the ascites tumor cell response to a heterologous antiserum. Cancer Research. **18:** 1179–1185.
23. Green, H., P. Barrow & B. Goldberg. 1959. Effect of antibody and complement on permeability control in ascites tumor cells and erythrocytes. J. Exptl. Med. **110:** 699–713.
24. Wilbrandt, W. 1948. Der kompensationstest der kolloidosmotischen hämolyse. Helv. Physiol. Acta. **6:** 234–246.
25. Van Allen, C. M. 1926. Volume measurement of blood platelets. J. Lab. Clin. Med. **12:** 282–285.
26. Lowry, O. H., N. J. Rosebrough, A. L. Farr & R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. **193:** 265–275.

27. Schmidt, G. & S. J. Thannhauser. 1945. A method for the determination of desoxyribonucleic acid, ribonucleic acid and phosphoproteins in animal tissues. J. Biol. Chem. **161:** 83–89.
28. Dische, Z. 1955. *In* The Nucleic Acids. **1:** 300–302. E. Chargaff and J. N. Davidson, Eds. Academic Press. New York, N. Y.
29. Bordet, J. 1939. Traité de l'Immunité. 2nd ed. : 370. Masson. Paris, France.
30. Bernheimer, A. W. 1947. Comparative kinetics of hemolysis induced by bacterial and other hemolysins. J. Gen. Physiol. **30:** 337–353.
31. Latta, H. & S. S. Polt. 1954. Sucrose inhibition of resorcinol hemolysis. Science. **120:** 271–273.
32. Colter, J. S., H. Koprowski, H. H. Bird & K. Pfeister. 1956. Immunological studies with protein fractions isolated from Ehrlich ascites carcinoma cells. Nature. **177:** 994–995.

Discussion of the Paper

Lawrence: Green and Goldberg have done something in an area that has provoked many who are concerned with hypersensitivity on the one hand and with the behavior of microbial cells on the other. They have adapted the techniques of Monod and his school to mammalian systems and, in a slower-growing cell population, have clarified the events that occur in the membranes of sensitized cells. I think this would also be the system par excellence to demonstrate an effect of a delayed allergen such as tuberculin on tuberculin-sensitive cells.

Some work with which Pappenheimer and I were concerned has a bearing on this. If leukocytes from tuberculin-sensitive individuals are incubated with antigen, the transfer factor activity appears in the supernatant and the cells are then desensitized. When these cells are spun out and examined by the usual methods, they appear to be intact and viable. I wonder what a system such as Green and Goldberg have introduced here might have revealed?

Zoltan Ovary: Until now very little attention has been given to what happens to the cell when all components of complement C′ were fixed.

The work of Mayer and his collaborators, and also of others, established very clearly the sequence and the kinetics by which different components of C′ fix on the sensitized erythrocyte in immune hemolysis. We know that when C′ reacts with the sensitized erythrocyte, the first to be fixed is the first component and then comes the fourth component. These steps require cations. Only when these two components have reacted can the second component get fixed, and this step requires magnesium ions. Finally, the two constituents of the third component react, and then the erythrocyte is in a state designated E STAR. For these last steps no cations are required. The reaction of the components of C′3, however, is more temperature-dependent than the former steps. It was believed until now that nothing can prevent the lysis of a cell in a state of E STAR. It was not well understood, however, why not all the E-STAR cells undergo immediate lysis. The experiments of Green and Goldberg explain perfectly what happens. The time for lysis would depend on the number of holes made in the membrane which, in turn, determines the rate of cation equilibration and consequent swelling.

These experiments also give a clue to what happens *in vivo* when cytotoxic antibody is injected. For example, it was shown long ago that when anti-Forssman antibody is injected into the guinea pig, an animal with the Forssman hapten in its cell, necrosis and death of the cell occurs. There is good evidence indicating that the action of this cytotoxic antibody leads C′ to cause necrosis and death of the cell.

With homologous antibody the pattern must be the same. In homotransplantation immunity, if cytotoxic antibody plays a role, as many believe it does, the mechanism may be the same as that described by Green and Goldberg.

A new field of investigation is opened by these experiments. It would be most interesting to know what happens to the cell if swelling and the outflow of proteins are prevented. Does it remain viable? Do the processes of metabolism continue in such a cell? Can it heal?

Howard Green: We have incubated these cells in the presence of a variety of proteins having nothing to do with the cells, that is, with pure proteins such as serum albumin and bovine gamma globulin for various periods of time; then we injected the cells into mice immunized against these proteins. We found that this had no effect at all on the ability of these cells to grow out in these mice.

With respect to bacteria, of course, the colloid osmotic pressure gradient between the inside of the cell and the outside is supported by a rigid cellular membrane: a rigid wall. When this wall is removed by any of a variety of known means, these bacteria must swell in the same way that animal cells swell. No mechanism of this sort exists in animal cells in which the cellular membrane is a purely passive structure and in which the colloid osmotic pressure gradient is compensated for by completely different means.

As to Ovary's questions: we have tried to see whether cells treated with antibody and complement in the presence of protective concentrations of protein might, if they were left in such protein for a long time, recover in some way, but thus far we find no evidence of their recovery.

These cells are nonviable when they are injected into mice, and they do not recover the state of cation impermeability that they had before being exposed to antibody and complement.

Part IV. Graft Versus Host Reactions and Immunological Tolerance

SOME EXPERIMENTS ON ACQUIRED IMMUNOLOGICAL TOLERANCE IN THE GOAT*

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The injection of a heterologous protein into a newborn animal prevents antibody formation to this particular antigen during part or all of the adult life of the animal.[1-12] This specific inhibition of antibody response is called immunological tolerance and is attributed to the effect's having been produced on the central antibody-forming mechanism.[13-15]

Several features of the response of adult tolerant animals to antigens deserve further study.[15] These include the specificity of tolerance to defined antigens and the persistence of tolerance in an adult animal. The latter is of particular importance for the development of any informed hypothesis on autoimmune disease as well as on the mechanism of immunological tolerence at the cellular level. The underlying question, whether the persistence of an antigen is required for the maintenance of the refactory state, is one that also has been posed in studies of antibody synthesis. If the induction of tolerance involves a modification of the synthetic mechanism, tolerance should persist—apart from mutations of antibody-forming cells—to the end of the life of the organism. However, if tolerance depended on the persistence of antigens at some locus of the cell, its duration would depend on the rate of division of the cell and on the concentration of antigen per cell.

Tolerance to human albumin in the rabbit can persist through the life of the animal; some rabbits, injected with as little as 20 mg. of human albumin, during the first few days after birth have maintained their immunologically refactory state for over four years. There is, however, also evidence that tolerance may be of much shorter duration[9,10] and that it might be reinforced by injection of the antigen during the animal's adult life.[16-18]

This paper, reporting that tolerance to human albumin may be induced in the goat, is presented with the intention of illustrating criteria that may have to be met before failure to induce tolerance or breakdown of established tolerance can be considered to have been conclusively proven.

Newborn goats (TABLE 1) were injected with human albumin (AP3) prepared by ether fractionation[19] at the Blood Products Unit of the Lister Institute, London, England. An intraperitoneal injection was administered immediately after birth of a goat or was given in 9 separate injections of 100 mg., beginning immediately after birth and thereafter at intervals of 24 hours.

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A second group of animals born during the same period was not injected at birth. Seven to 11 weeks after birth the animals of both groups were given a series of 3 intravenous immunizations; they were bled 12 days after the last injection. Five weeks after the last injection a second course of immunizations was carried out, and the animals were bled 5 days after the end of this immunization (TABLE 2). The sera obtained were examined for antibody by two methods: by the agglutination of tanned erythrocytes sensitized with human albumin,[5,7,20] and by quantitative precipitin assays. Data obtained by agglutination assay showed no difference in antibody titer between animals injected at birth and animals not so injected (TABLE 3). On the other hand, quantitative precipitin assays led to data indicating a completely different response of the two groups of goats: animals injected at birth did not have any detectable antibody, while every one of the animals not injected at birth had synthesized precipitable antibody.

TABLE 1

GOATS INJECTED AT BIRTH AND CONTROLS

No.		78	79	83	84	85	86	87	88	90	93	94	95
Relationship		littermates		littermates		littermates							
				common paternal animal									
Weight at birth (kg.)		2.8	2.2	3.5	3.6	3.3	3.2	4.6	7.3	—	—	—	—
Inject. immediately after birth	No. of days after birth	0–8	—	0–8	0–8	0	—	0	0	—	—	—	—
	Human albumin (mg.)	9 × 100	—	9 × 100	9 × 100	1 × 900	—	1 × 900	1 × 900	—	—	—	—
Age at which "adult" injections were started (days)		81	81	68	68	55	55	49	47	47	46	46	46

TABLE 2

INTRAVENOUS INJECTIONS DURING "ADULT" LIFE OF GOATS

	Course		
	First	Second	Third*
Days of injections†	1, 3, 5	33, 35, 37	89
Quantity injected (mg.)	3 × 10	3 × 15	1 × 1,000
Day of bleeding†	17	42	Regular intervals for 25 days

* Only animals injected at birth (see FIGURE 4).

† Counting as 1 the day of the first injection during "adult" life; for actual age of animals, see TABLE 1.

It seemed possible that the animals injected at birth had responded to human albumin, but that the resulting antibody was not of the precipitating kind and therefore remained undetected by the precipitin test.

Some nonprecipitating antibodies can be coprecipitated by potent precipitating antibody directed against the same antigen. All sera were, therefore, examined by a technique of coprecipitation. A potent precipitating serum was selected and mixtures were prepared, each containing an equal quantity of the standard serum and of one of the sera either from animals injected at birth or from the controls. The quantitative precipitin curve of each of these mixtures with AP3 was determined (FIGURE 1). The results confirmed those obtained with the direct precipitin test. Animals injected at birth did not contain any antibody detectable by the coprecipitin test; animals not so injected contained between 14 and 438 μg. nitrogen per ml. of serum (TABLE 4).

TABLE 3

RECIPROCAL AGGLUTINATION TITERS (TANNED ERYTHROCYTES SENSITIZED WITH HUMAN ALBUMIN) OF GOATS INJECTED AT BIRTH AND OF CONTROLS

No. animal	Twelve days after first course	Five days after second course
Injected at birth		
78	1000	20,000
83	500	2000
84	<10	200
85	<10	<10
87	1000	2000
88	<10	200
Controls		
79	200	50,000
86	<10	10,000
90	200	20,000
93	200	10,000
94	200	2000
95	<10	2000

How could the different results obtained by the two different tests be reconciled? A choice had to be made between the following two hypotheses:

(1) The injections at birth had not inhibited antibody formation, but had modified the antibody-forming mechanism in such a fashion that antibody was still being formed but had a dissociation constant with respect to antigen that led to highly dissociable complexes of antigen and antibody. The resulting antibody-antigen complex was so highly dissociable that the antibody, while still agglutinating sensitized and tanned cells, could not precipitate free antigen from solution even in the presence of antibody that firmly bound antigen. In other words, any aggregate of antigen and mixed antibody that incorporated the dissociable antibody would immediately break at the points at which the dissociable antibody was incorporated into the aggregate; thus coprecipitation would be impossible.

(2) An alternative mechanism, leading to the described results, could arise from the presence of an antigenic impurity and the formation of antibody to this impurity. In the protein injected at birth the impurity would be present in such small quantities as not to induce tolerance or so as to affect only

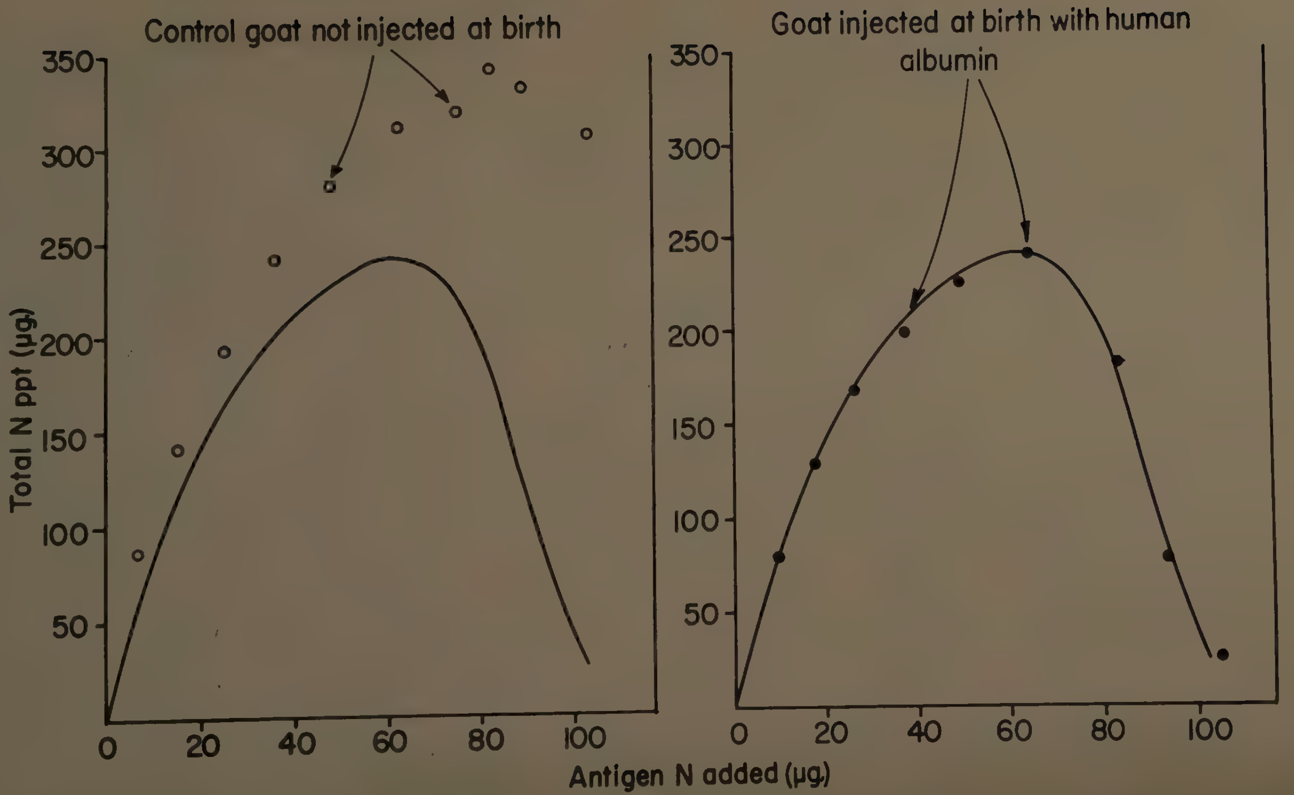

FIGURE 1. Coprecipitin test. Sera from immunized goats are mixed with a standard serum; the quantitative precipitin curve of this mixture is compared with that of the standard serum mixed with serum from normal goats. Key: —, standard serum mixed with an equal volume of the serum of a normal goat; ○, standard serum mixed with an equal quantity of the serum of a control goat immunized with human albumin; ●, standard serum mixed with an equal volume of the serum of a goat injected at birth and subsequently "immunized" with human albumin.

a portion of the cells involved in antibody formation. In the course of immunizations during adult life an antibody to this impurity would be formed.

The relatively high titers that the animals injected at birth showed after the second course of immunization (TABLE 3) induced us to consider the second of these hypotheses as the less likely and to examine initially the first hypothesis.

The sensitivity of the agglutination test is much greater than that of the precipitin test. Was it possible that the discrepancy between the results obtained with these two tests was due to the very much greater sensitivity of the agglutination test and that we were simply detecting a different order of magnitude of antibody concentration with the agglutination test than with that of the precipitin assay? Several independent assays, comparing the sera of controls and of animals injected at birth, confirmed the relative potencies of the sera from both groups and confirmed their similarity (TABLE 3). Nevertheless, the relative sensitivity of agglutination and precipitin assays still could be invoked if the agglutination titer were not a linear function of antibody concentration or if antibody nitrogen and agglutination titer were not proportional to one another. These two points had to be examined before the trivial explanation of the discrepancies between our two tests could be ruled out. FIGURES 2 and 3 show such experiments and indicate quite clearly that the relationships are approximately linear. Hence the relative sensitivities of the two tests employed could not be answerable for the observations under discussion.

TABLE 4

ANTIBODY N/ML. OF SERUM DETERMINED BY COPRECIPITIN TEST

Bleeding after Second Course of Immunization

No. goat	Serum (μg. N/ml.)
Injected at birth	
78	No detectable antibody N
83	
84	
85	
87	
88	
Controls	
79	438
86	158
90	98
93	124
94	41
95	14

If antibody is produced in small quantities and for a short period of time, it may be completely consumed by removing the foreign antigen remaining in the circulation. It could not be demonstrated by conventional tests, but could be detected by studying the elimination of antigen from the circulation. We, therefore, proceeded to examine the elimination of human albumin from the circulation of the goats injected with human albumin at birth. The antigen concentration in sera was measured by immunochemical methods. Each serum was treated as a source of antigen, and precipitation curves were determined, employing the sera containing human albumin as the antigen, and a standard serum from a goat immunized with human albumin as the antibody. The curves so obtained were compared with curves prepared against known quantities of human albumin dissolved in normal goat serum. The antigen content of a serum was thus determined as the average dis-

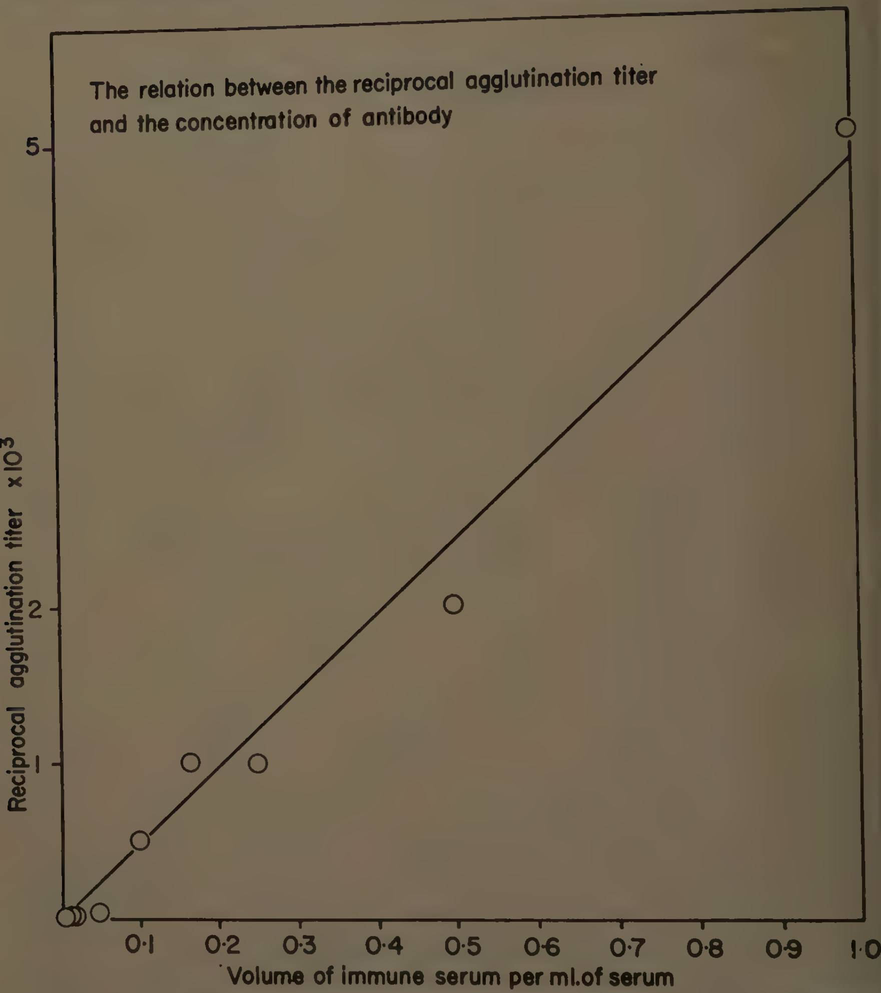

FIGURE 2. Agglutination titer as a function of concentration. The relation between reciprocal agglutination titer and concentration of human albumin antiserum (goat) is linear. The human albumin antiserum was diluted with normal goat serum, and the agglutination titer of the resulting mixture was measured with tanned sheep erythrocytes sensitized with human albumin (AP3). Key: —○—, reciprocal agglutination titer.

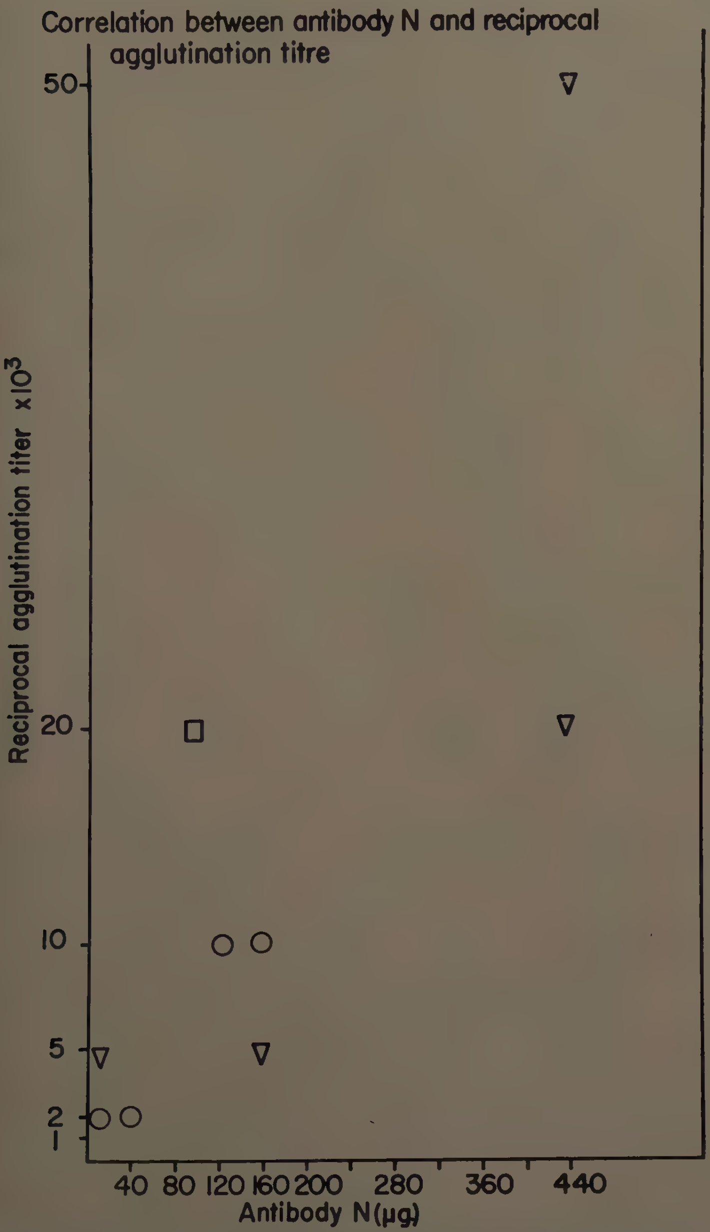

FIGURE 3. Antibody nitrogen and agglutination titer. The content of antibody N/ml. of 6 human albumin antisera from goats is correlated with the reciprocal agglutination titer of the sera (tanned sheep erythrocytes sensitized with human albumin (AP3)). Key: □, 1 determination of agglutination titer; ○, 4 determinations of agglutination titer; ▽, 4 determinations of agglutination titer.

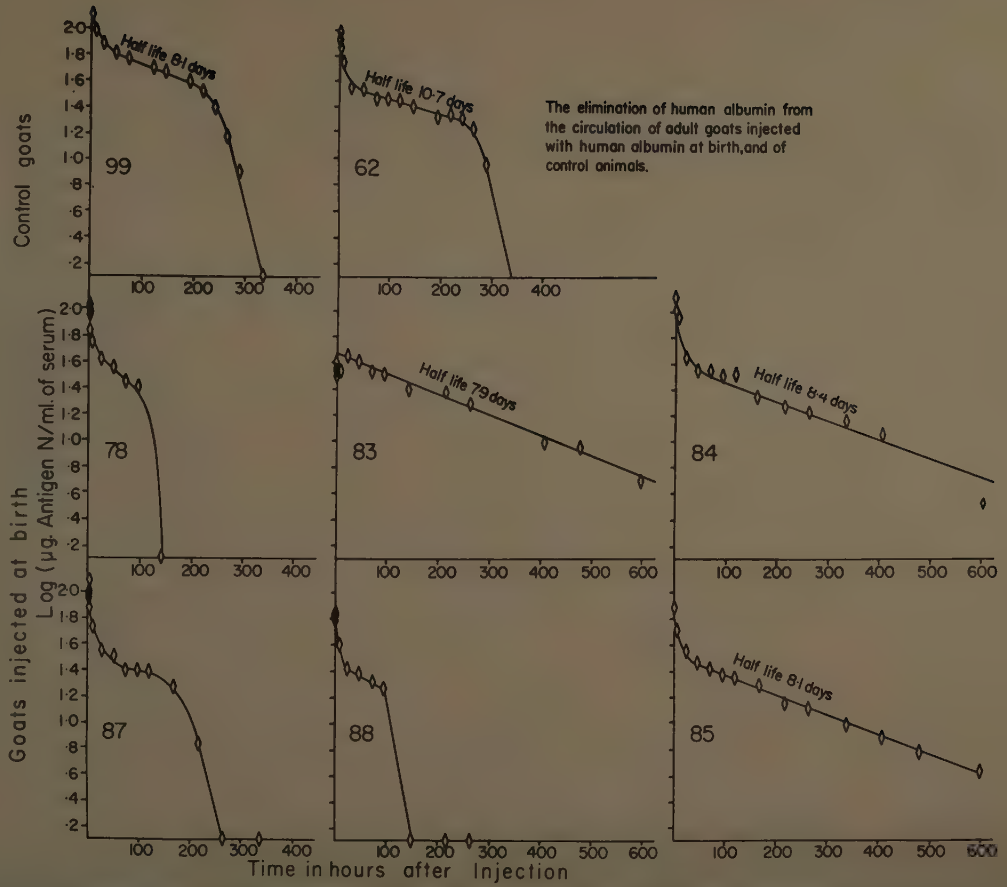

FIGURE 4. Removal of antigen from the blood stream. The elimination of human albumin preparation (AP3) from the circulation of adult goats that have not been injected with human albumin prior to this experiment (99, 62) and of adult goats that were injected at birth and received 2 courses of injections (*See* TABLE 2) before the administration of the antigen of which the elimination is being measured (78, 83, 84, 87, 88, 85). Key: –◇–, concentration of human albumin measured by quantitative precipitin assay.

placement between two quantitative precipitin curves. The results of these measurements are shown in FIGURE 4. In a normal adult animal (*see* top of FIGURE 4) human albumin disappears from the circulation in three phases: the first, showing a rapid rate of change in concentration, is due to distribution of the antigen between the blood stream and other tissue spaces; the second, showing a relatively slow and steady rate of elimination, is due to breakdown of the antigen by metabolic processes; the third phase, during which antigen is removed very quickly, is due to the appearance of antibody. In 3 goats, injected with AP3 at birth, antigen was eliminated in two phases.

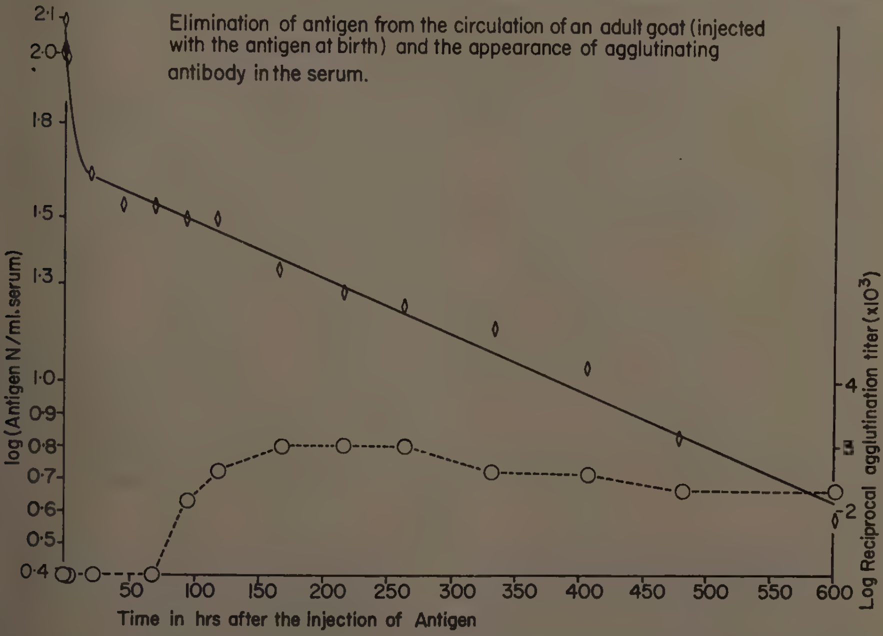

FIGURE 5. Simultaneous presence of antigen and antibody. The simultaneous presence of antigen and antibody in the circulation of an adult goat injected with human albumin preparation (AP3) at birth. Key: —◇—, concentration of antigen in the circulation (determined by quantitative precipitin assay); – –○– –, reciprocal agglutination titer determined with tanned sheep erythrocytes sensitized with AP3.

In other words, the heterologous antigen was eliminated as if it were homologous; 3 other goats showed the third phase of rapid elimination characteristic of an antibody response. In all instances, however, circulating antibody could be demonstrated by agglutination of tanned sensitized red cells in the blood stream when antigen was still present (FIGURE 5). This seemed to indicate the presence of antigen-antibody complexes in the circulation and to support the view that animals injected at birth produced an antibody that gave a highly dissociable complex with antigen.

Before this hypothesis could be accepted, it was necessary to examine the alternative hypothesis and to prove that the antigen in the circulation was identical with the antigen against which the antibody was directed. This could be achieved by inhibiting the agglutination reaction. This technique, previously used in examining the specificity of acquired immunological tolerance,[5,7] consists in adding soluble antigen to serum prior to agglutination. The agglutinating potency of the serum thus absorbed is less than that of the untreated serum if the soluble antigen with which the serum is absorbed is identical with the antigen on the red cells with which the agglutinating antibody interacts. If such a condition is fulfilled, the agglutinating potency decreases as the quantities of the antigen, which are added prior to agglutination, increase. An antibody directed to human albumin would thus be inhibited by human albumin; different preparations of human albumin, containing more than 90 per cent of this protein, should be equally effective as inhibitors. If, on the other hand, antibody were directed, not to human albumin, but to an antigenic impurity, different preparations of human albumin would not necessarily be equally inhibitory. Preparations obtained by different techniques probably would not contain impurities in the same quantities and therefore would differ considerably in their powers to inhibit. Clearly, only positive results could have any evidential value. A negative result would leave open the possibility that the antigenic impurities were present in the same proportion in the two albumin preparations; an explanation that would become less likely as more albumin preparations, fractionated by different methods, were utilized.

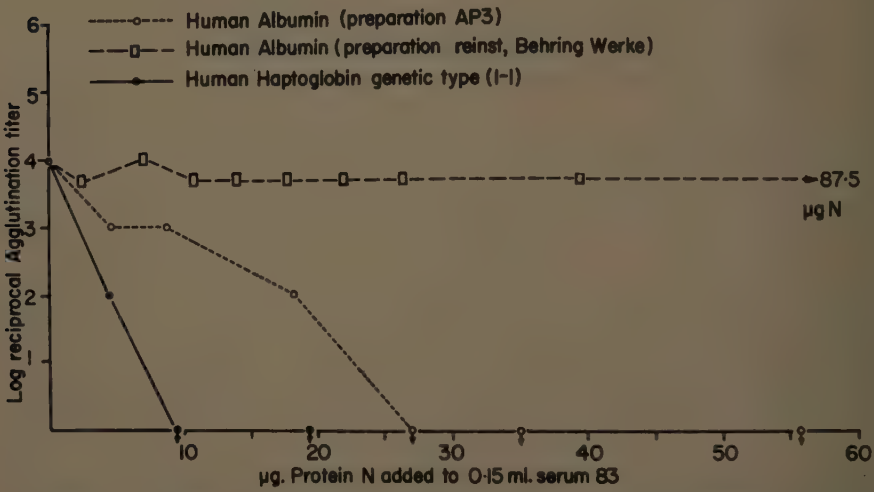

FIGURE 6. Antibody of goats tolerant to human albumin. The specificity of the antibody found in the serum of a goat injected with human albumin (AP3) at birth and subsequently immunized with the same antigen. Inhibition by various protein preparations of the agglutination of red cells sensitized with human albumin (AP3). (Serum 83, third course; see TABLE 2.)

Sera obtained at the end of elimination experiments were the first object of inhibition tests; the inhibitory power of preparation AP3 and of albumin *reinst* obtained from the Behringwerke (Marburg a/L, German Federal Republic) were compared (FIGURE 6). There was a marked difference in inhibiting potency between these two preparations. Experiments with other preparations of albumins and with alpha-globulin preparations finally showed that haptoglobin was the most effective inhibitor. It thus seemed likely that the antibody in animals injected at birth was directed against a protein that was present in much higher concentrations in preparations of haptoglobin than it was in preparations of human albumin. This conclusion remained tentative until the possibility was eliminated that inhibition was due to displacement of human albumin from the sensitized tanned red cells rather than to inhibition of antibody. The following events could be visualized: an antigen B added to serum would not combine with it; when the serum containing antigen B was added to tanned and sensitized red cells, antigen B would displace human albumin from the red cells. As a result, the red cells would no longer be agglutinated by an antibody to human albumin. Were these events to occur, the observed results would be little different from those obtained when antigen combined with antibody and rendered the antibody incapable of combining with human albumin attached to red cells. The test for displacement was simple. Erythrocytes were sensitized with AP3, labeled with radioactive iodine. The sensitized cells were washed and incubated with diluted normal goat serum to which AP3 was added, with diluted normal goat serum to which haptoglobin was added and, for control, with diluted normal goat serum. No difference could be found between the three sets of cells treated with these preparations. It therefore was reasonable to conclude that displacement did not occur and that the inhibition experiments showed that antibody in the sera of animals injected at birth was directed against a protein other than human albumin (TABLE 5). It remained to examine the inhibition of sera of all tolerant animals and to com-

TABLE 5

ADDITION OF HUMAN ALBUMIN (AP3) AND OF HUMAN HAPTOGLOBIN TO RED CELLS TANNED AND SENSITIZED WITH I^{131}-HUMAN ALBUMIN (AP3)*

Protein added to tanned sensitized red cells		Molecules of I^{131}-protein (AP3) per tanned and sensitized red cell after incubation with unlabeled protein (× 10^7)
Protein	Weight in cell suspension (μg./1.5 ml.)	
Human albumin (AP3)	133	5.5
	266	4.7
	666	6.1
	1350	6.3
Human haptoglobin	33	5.1
	66	5.6
	166	5.1
	330	5.5
None	0	4.7

* Tanned red cells, 0.05 ml. (2.5 v/v), sensitized with labeled human albumin [1.08 × 10^8 molecules of iodinated albumin (AP3) per red cell], were incubated for 2 hours at 37° C. in 10 per cent normal goat serum (decomplemented and absorbed with sheep erythrocytes) in the presence of varying amounts of unlabeled human protein preparations.

pare it with inhibition of sera from the controls by employing the protein preparations that had been found to discriminate between antibodies to human albumin and antibodies to an impurity. These experiments, with bleedings following the second course of immunization, showed conclusively that the serum of animals not injected at birth contained antibody to human albumin and that the serum of 5 of the 6 animals injected at birth did not contain demonstrable antibody to human albumin (FIGURE 7*A* and *B*).

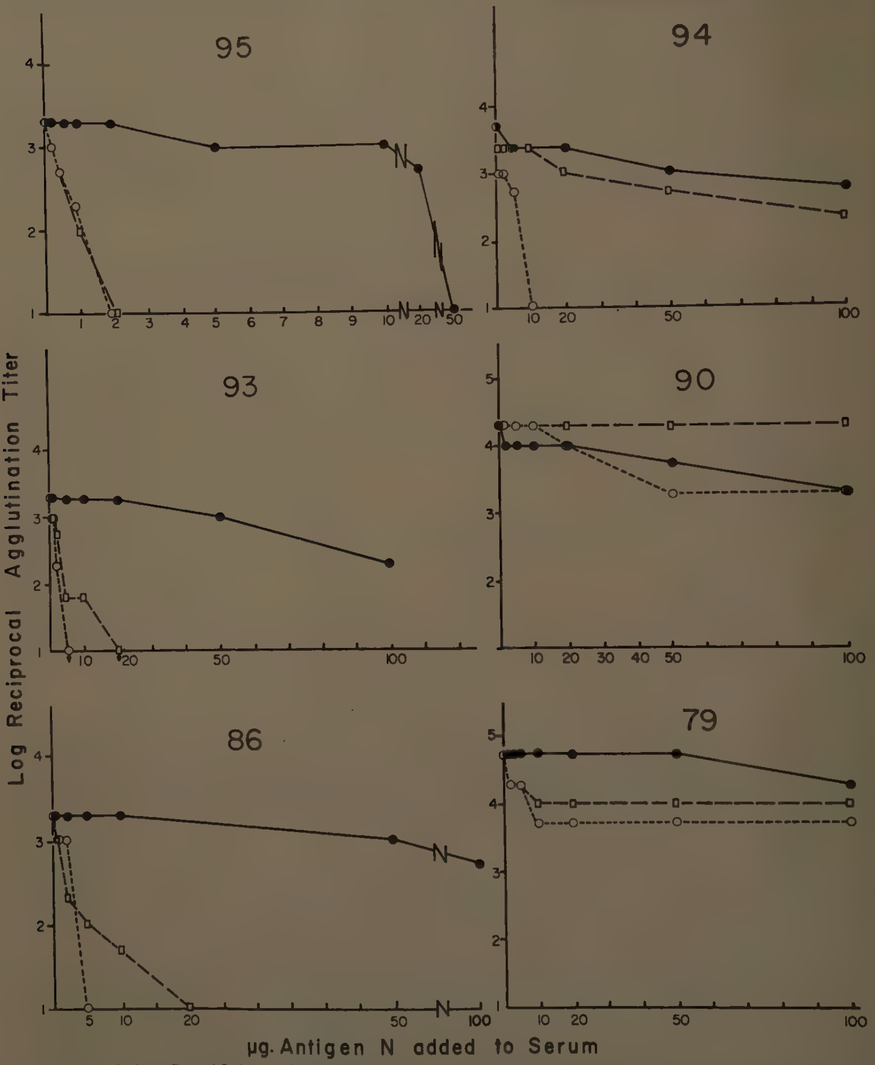

FIGURE 7*A*. Specificity of antibodies in the sera of controls. Inhibition by various protein preparations of the agglutination of red cells sensitized with human albumin (AP3) by sera (second course; *see* TABLE 2) from *control* animals (not injected at birth) injected during adult life with AP3. Key, protein preparations used for inhibition: – –○– –, human albumin (preparation AP3); – –□– –, human albumin (preparation *reinst*, Behringwerke); —●—, human haptoglobin (preparation A).

Nevertheless, antigen had been eliminated from the circulation of 3 of the goats injected at birth with a third rapid phase characteristic of antibody formation. Although this indicates that antibody is being formed, it does not imply necessarily that the antibody is directed against human albumin.

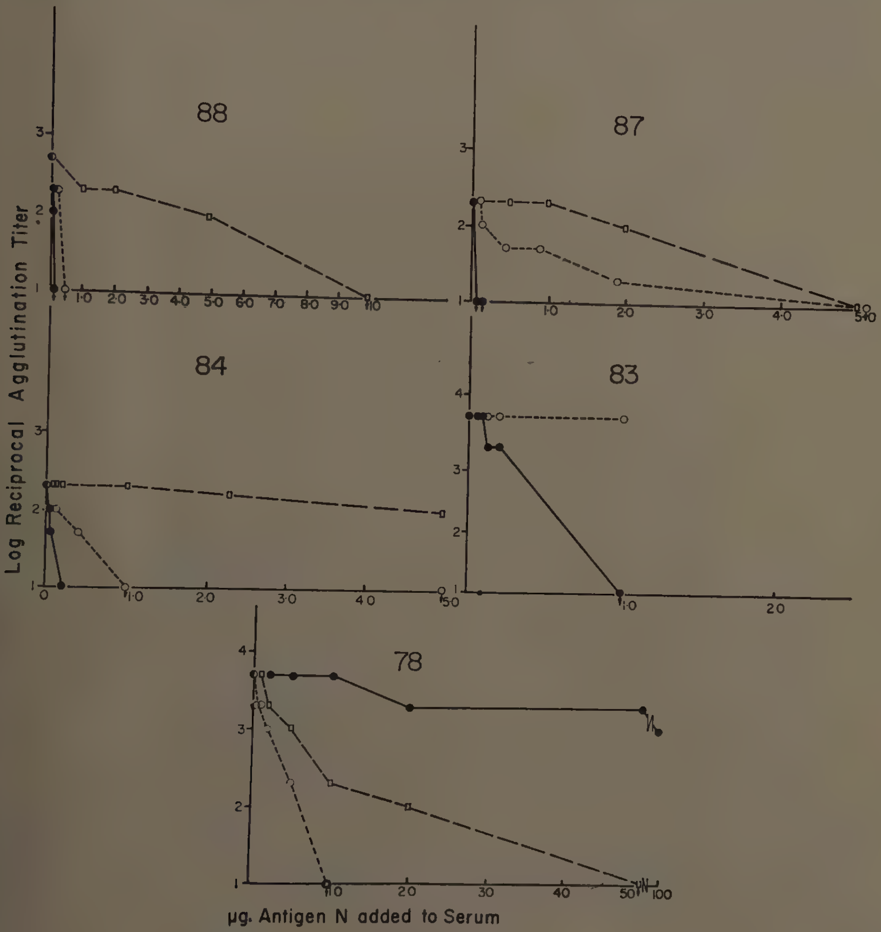

FIGURE 7*B*. Specificity of antibodies in the sera of goats injected with human albumin at birth. Inhibition by various protein preparations of the agglutination of red cells sensitized with human albumin (AP3) by sera (second course; *see* TABLE 2) from animals *injected at birth* and during adult life with AP3. Key, protein preparations used for inhibition: – –○– –, human albumin (preparation AP3); – –□– –, human albumin (preparation *reinst*, Behringwerke); —●—, human haptoglobin (preparation A).

If two antigens are being eliminated at the same time, their relative rates of disappearance from the blood stream during the first phase of elimination and particularly during the second phase of metabolic breakdown, could be quite different. Antibody formation starts several days after the injection. By that time the relative composition of the two antigens could be quite different from that of the mixture originally injected. It would thus be possible that at the time of the third phase of elimination (by antibody) only one of the two antigens remains in the circulation and that the elimination by antibody of this remaining antigen is being observed. This possibility can be excluded if it can be shown that the percentage of heterologous antigen N left in the blood stream at the start of elimination by antibody is greater than the quantity of the second antigen in the protein preparation originally injected. It was shown that this condition is not fulfilled and that the observed elimination of antigen is due to the removal of human albumin from the 3 goats under observation. Thus it has been established that tolerance was induced in 3 of 6 goats injected at birth.

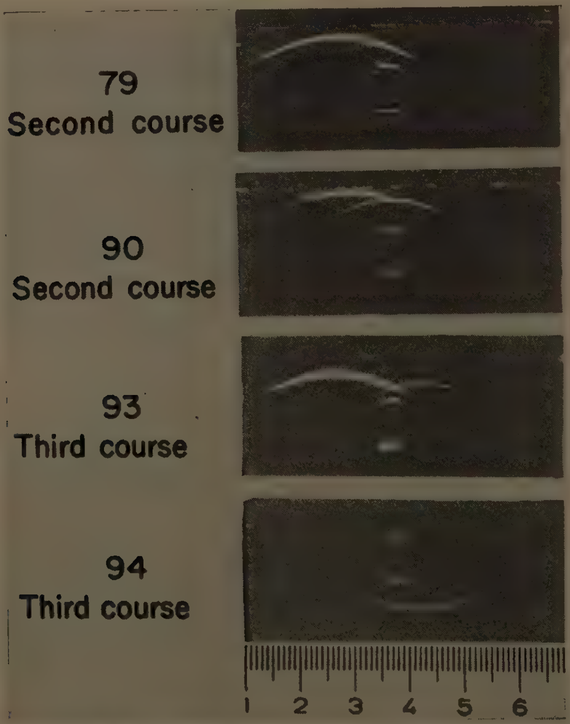

FIGURE 8. Antibodies to albumin, α and α_2 globulin found by immunoelectrophoresis in the sera of goats (controls) immunized with human albumin (AP3).

For reasons given above, some indication was sought as to the nature and quantity of the impurity in AP3 to which antibody was synthesized by animals tolerant to human albumin. Earlier experiments with AP3 have shown that this antigen contains an α_1 and an α_2 globulin. Some sera from immune goats showed, on immunoelectrophoresis,[5,21] an antibody to one of them (FIGURE 8); none of the animals injected at birth showed precipitating antibodies to any antigen. Experiments already described suggested haptoglobin as the impurity to which goats, tolerant to human albumin, had made an antibody. This was tested by comparing the hemoglobin-binding capacity[22] of the haptoglobin preparation with that of albumin preparation AP3 and by comparing the agglutinating-inhibiting capacity of these two protein preparations with serum 83, which had been shown to be devoid of antibody to albumin. In terms of the first of these comparisons, AP3 contained 5 per cent haptoglobin and, in terms of the second, it contained 7 to 8 per cent haptoglobin. These values are sufficiently similar to render it likely that the impurity to which tolerant animals have made antibody was directed against haptoglobin (FIGURE 9). The percentage is sufficiently low to suggest that the third phase of elimination after the third course of immunization, observed in 3 goats injected at birth, represents the removal of human albumin from the circulation and not the removal of a macromolecular impurity.

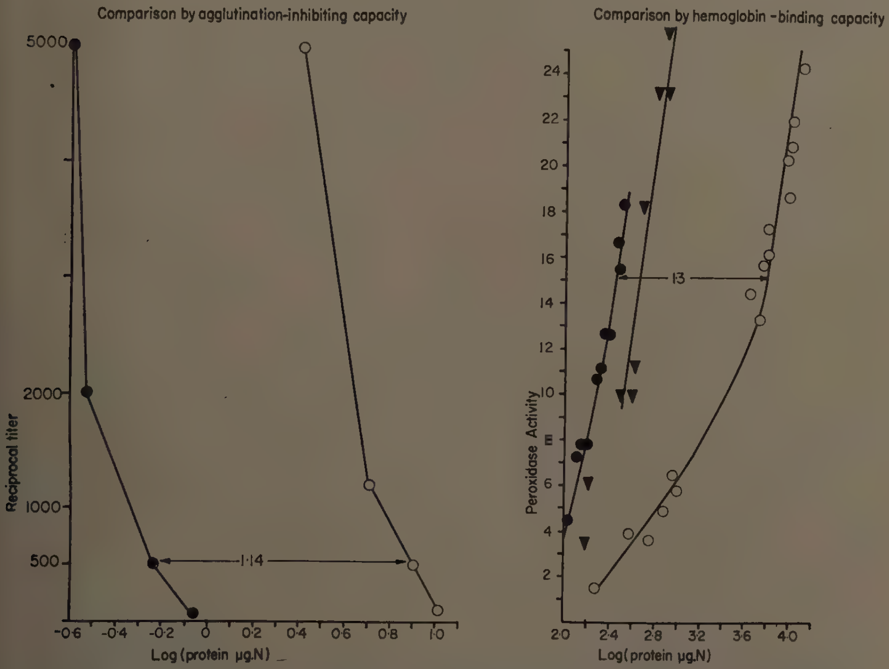

FIGURE 9. Haptoglobin in human albumin preparation (AP3). The comparison of human albumin (preparation AP3) and of human haptoglobin in terms of the agglutination-inhibiting capacity (*left*) and in terms of their capacity to bind hemoglobin (*right*). Key, protein preparations used: ●, human haptoglobin (preparation A); ▼, human haptoglobin (preparation B); ○, human albumin (preparation AP3).

The experiments described above have shown that tolerance to human albumin may be induced in goats by the injection of the antigen into the newborn animal. An antigenic impurity in the albumin preparation injected did not induce tolerance, but induced antibody formation after injection of the adult animal. As a consequence, antibody could be detected in animals tolerant to human albumin and even could be found in the circulation at the same time as antigen. The observation that the antibody was directed against an antigen other than that remaining in the circulation indicated the presence of the antigen impurity that was finally shown to be haptoglobin.

Few preparations of purified proteins will be free of antigenic contaminants; the situation that has been analyzed therefore could be found in other animals and with other purified proteins as antigens. The smaller the percentage of the antigenic impurity, the smaller the likelihood that the quantity of protein injected into neonatal animals will contain the impurity in sufficient amount for tolerance to be induced to the impurity, and the greater will be the likelihood that the impurity will provoke an antibody response after prolonged immunization of the adult animal. Consequently, the appearance of antibody, after prolonged immunization of an adult animal injected at birth, cannot necessarily be taken as proof that tolerance has been broken. Such a conclusion must depend on evidence that the antibody is directed against the principal antigenic component to which tolerance was originally established.

The problem is even more complex when cells are used as antigens. In such case, the agent to which tolerance is induced and by which it is tested consists of a large number of different antigens, all acting independently in inducing tolerance and all present in different relative quantities.

If homologous cells that can propagate in the recipient are injected, the supply of all antigens may be sufficient for tolerance to be induced. Cells that cannot propagate and are heterologous may present a different problem. The antigens present in very low concentration on the surface of such cells would not induce tolerance. More abundant antigens would induce tolerance. Subsequent immunization would lead to the formation of antibody to the "rare" antigens only. It might be concluded that tolerance had not been induced (which is correct on the cellular but not on the antigen level). However, the antibody may interact with the cell in a manner very different from antibodies of immune animals not injected with the cell at birth. The latter antibodies would be directed to the abundant as well as to the rare antigens, and the effect of interaction with the rare antigens would be masked by the effect of interaction with the abundant antigens. Consequently, it might appear that the dissociation constant or valency of the antibody has been affected by neonatal injection. However, the dissociation constant of the antibody may have remained unaffected, while the distribution of specificities had been altered. In other words, antibodies of animals injected at birth would react with only some of the areas of the cells, while antibodies from animals not so injected would interact with many more antigens and, in particular, with the more abundant antigens on the surface of the cell.

Acknowledgments

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References

1. Hanan, R. & J. Oyama. 1954. Inhibition of antibody formation in mature rabbits by contact with the antigen at an early age. J. Immunol. **73:** 49–53.
2. Kerr, W. R. & M. Robertson. 1954. Passively and actively acquired antibodies for *Trichomonas foetus* in very young calves. J. Hyg. **52:** 253–263.
3. Kerr, W. R. & M. Robertson. 1956. Partial immunological paralysis to *Trichomonas foetus* antigen in calves. J. Hyg. **54:** 415–418.
4. Dixon, F. J. & P. H. Maurer. 1955. Immunological unresponsiveness induced by protein antigens. J. Exptl. Med. **101:** 245–257.
5. Cinader, B. & J. M. Dubert. 1955. Acquired immune tolerance to human albumin and response to subsequent injections of diazo human albumin. Brit. J. Exptl. Pathol. **36:** 515–529.
6. Cinader, B. & J. M. Dubert. 1956. Specific inhibition of response to purified protein antigens. Proc. Roy. Soc. London. **B146:** 18–33.
7. Cinader, B. & J. H. Pearce. 1958. The specificity of acquired immunological tolerance to azo proteins. Brit. J. Exptl. Pathol. **39:** 8–29.
8. Cinader, B., J. H. Pearce & B. G. Carter. 1958. Acquired immunological tolerance to bovine ribonuclease. Nature. **181:** 1208–1209.
9. Wolfe, H. R., C. Tempelis, A. Mueller & S. Reibel. 1957. Precipitin production in chickens. XVII. The effect of massive injections of bovine serum albumin at hatching on subsequent antibody production. J. Immunol. **79:** 147–153.
10. Terres, G. & W. L. Hughes. 1959. Acquired immune tolerance in mice to crystalline bovine serum albumin. J. Immunol. **83:** 459–467.
11. Chase, M. W. 1959. Immunological tolerance. Ann. Rev. Microbiol. **13:** 349–376.
12. Bussard, A. 1957. Tolérance immunologique provoquée chez le lapin envers certains antigènes de la levure. Compt. rend. acad. sci. **245:** 2430–2432.
13. Billingham, R. E., L. Brent & P. B. Medawar. 1953. ‘Actively acquired tolerance’ of foreign cells. Nature. **172:** 603–606.
14. Billingham, R. E., L. Brent & P. B. Medawar. 1955. Acquired tolerance of skin grafts. Ann. N. Y. Acad. Sci. **59**(3)**:** 409–416.
15. Billingham, R. E., L. Brent & P. B. Medawar. 1956. Quantitative studies on tissue transplantation immunity. III. Actively acquired tolerance. Trans. Roy. Soc. London. **B239:** 357–414.
16. Smith, R. T. & R. A. Bridges. 1958. Immunological unresponsiveness in rabbits produced by neonatal injection of defined antigens. J. Exptl. Med. **108:** 227–250.
17. Smith, R. T. 1958. Duration of immunological unresponsiveness resulting from neonatal injection of defined antigens in rabbits. Federation Proc. **17:** 535.
18. Cohen, C. 1959. Anamnestic reactions in rabbits showing unresponsiveness to erythrocytic antigens. Transplantation Bull. **6:** 426–428.
19. Kekwick, R. A. & M. E. Mackay. 1954. The separation of protein fractions from human plasma with ether. Med. Research Council (Brit.) Spec. Rep. Ser. No. **286.**
20. Boyden, S. V. 1951. The adsorption of proteins on erythrocytes treated with tannic acid and subsequent hemagglutination by antiprotein sera. J. Exptl. Med. **93:** 107–120.
21. Grabar, P. & C. A. Williams. 1953. Méthode permettant l’étude conjuguée des propriétes electrophorètiques et immuno-chimiques d’un mélange de protéines. Application au sérum sanguin. Biochim. et Biophys. Acta. **10:** 193–194.
22. Connell, G. E. & O. Smithies. 1959. Human haptoglobins: estimation and purification. Biochem. J. **72:** 115–121.

Discussion of the Paper

Jonathan W. Uhr: I congratulate Carter and Cinader on their extremely thorough and excellent study of this problem. Actually, Cinader’s presentation has been so clear and his discussion so thorough that there is only one comment I desire to make.

Despite the authors' use of highly purified antigens and precise quantitative techniques, their initial results obtained by using two different types of antibody assay gave apparently contradictory results. It was only through the use of further elegant techniques such as immunoelectrophoresis and the use of various serum fractions as inhibitors of hemagglutination reactions that they were finally able to demonstrate conclusively that antibody production to a minor impurity had clouded the initial results.

In studies of immune responses such as the delayed type of hypersensitivity and the capacity to reject homotransplants, there is no quantitative *in vitro* assay, and the materials used, as Cinader has mentioned, are frequently highly complex, consisting of literally dozens of antigens.

The inherent problems of immunological investigations are, of course, tremendously multiplied in such complex systems. It would seem reasonable to suggest that much of the discrepancy between results of various studies of cellular immune systems might well be traced simply to quantitative differences or to differences in the roles played by minor antigenic constituents. For example, the failure of newborn rabbits to develop tolerance after injection of leukocytes, as reported yesterday by Harris, might be simply the reflection of an inability to produce tolerance to minor antigenic components of the leukocytes.

I think it would be well worth a major effort to try to develop systems in the field of cellular immune responses that would lend themselves to more precise analytical study; the papers just presented show that such an endeavor is under way.

HILARY KOPROWSKI (*The Wistar Institute, Philadelphia, Pa.*): Is it possible to detect some of these impurities by other than immunological means? Would electrophoretic patterns show that there is some impurity in the albumin fraction?

A second question: Is this, by any chance, a particular action of the species of animal used for immunization? Or can this same pattern occur in other animals?

CINADER: In answer to the first question: we have been provided with electrophoretic analysis by the people who made the protein for us—the AP3 albumin prepared by ether fractionation. These people decided that the protein was homogenous and it certainly appeared homogenous to us when we looked at the electrophoretic patterns.

We knew from experiments with immunoelectrophoresis, which is much more sensitive, that this protein contained an α_1 and an α_2 globulin. We now believe that these two impurities, in fact, did not play any part.

In answer to your second question: we have used the same protein preparation in our experiments with rabbits. In these experiments, we did not find antibodies to these α_1 and α_2 globulins, although we had looked very carefully for them.

An identification of the haptoglobin at that stage would have been quite impossible, and it was rather fortunate that the work reported on haptoglobin in other laboratories—largely, of course, the work of Connell and Smithies—had reached the stage where we could use the information they had made available in the literature. There certainly is a species element in this, too, for I am convinced that our writers did not have an antibody to haptoglobin.

Silvers: I am curious. May I ask Cinader why he used goats?

Cinader: That is a very good question. We used goats because they were large animals. As you know, we have great interest in the specificity of immunological tolerance. We have worked with rabbits, injecting them with albumin and then immunizing them with human albumin (H.A.) later, and we got antibodies that seemed to be almost entirely adapted to the eso group.

The trouble with this work was that every time we desired to attempt really serious work we found that we had run out of the serum. Therefore, we decided to employ larger animals. Of course, we immediately thought of a whale, but we could not get a whale. On the other hand, a large goat herd was available, and we drew upon it.

ON THE ACQUISITION OF TOLERANCE BY ADULT CELLS*

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The phenomenon of acquired tolerance historically is connected very firmly with the prenatal or neonatal stage of development of an organism. Of course, it is fully legitimate to speak theoretically of the acquisition of tolerance in adult organisms. The question is whether such can occur and, if so, how to demonstrate it.

The chief purpose of this paper is to discuss the ways by which acquired tolerance may be demonstrated, not so much in the integrated organism, as usual, but rather in a population of spleen cells. It has been asked sometimes whether anyone ever has seen a tolerant cell. I think the answer is "No." However, I think that I have seen at least some isolated spleen cell populations that behaved in a tolerant way and in accordance with all classic requirements. Among such cell populations there have been apparently tolerant cells whose origin was in adult spleens. However, before coming to these questions, I shall discuss a few points of analytic technique.

The basic technique employed is the graft-versus-host assay (GVHA), described two years ago (Simonsen *et al.*, 1958), which is based on the fact that spleen and liver enlargement are constant and conspicuous signs in a rather early stage of the "runt disease," being especially pronounced about ten days after grafting (Simonsen and Jensen, 1959).

There are several reasons why the GVHA often is performed with litters of F_1 hybrids:

(1) Hybrid vigor usually ensures litters of good size and vitality.

(2) The litters may be used at least 10 days after birth instead of 1 day, which is approximately the end point for use of litters not genetically tolerant of the graft.

(3) They may be used to discriminate between immunologically competent cells of different genetic derivation; this is because 4- to 10-day-old hybrids are sufficiently mature to eliminate foreign cells by the host response to the graft and at the same time are young enough to allow a typical graft-versus-host reaction to develop when they are injected I.P. with adult cells from one of their parental strains (TABLE 1).

A warning is necessary here to those interested in using this method: it may fail entirely if the foreign graft (C in TABLE 1) belongs to the same H-2 group as the parental graft. Thus, if AKR spleen is grafted to (C3H × DBA/2), it gives as much splenomegaly as if adult C3H spleen had been grafted instead (AKR and C3H carry both the $H\text{-}2^k$ allele, while DBA/2 is $H\text{-}2^d$).

Apart from being an undesirable restriction in the applicability of the method, this finding proves that complete genetic tolerance of the graft is not required for the development of runt disease. It fits very well with the dem-

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onstration by Billingham and Brent (1959) that complete acquired tolerance also is not necessary in this respect.

Let us now approach the demonstration of tolerance in a spleen cell suspension. If we accept the fact, which we reasonably can do, that the splenomegaly in a GVHA is proof of immunological competence on the part of the grafted cells, how is tolerance in the graft then to be demonstrated?

In complete analogy with the definition of tolerance in the whole organism we must suppose that the cells, because of previous contact with a foreign antigen, have lost their ability to react against the very same antigen and yet have retained their normal reactivity to other antigens.

TABLE 1

DISCRIMINATION BETWEEN IMMUNOLOGICALLY COMPETENT CELLS OF DIFFERENT GENOTYPES BY THE GRAFT-VERSUS-HOST ASSAY*

Recipient	Donor	Splenomegaly
4 to 10 days old $(A \times B)F_1$	A	++
4 to 10 days old $(A \times B)F_1$	C	±
4 to 10 days old $(A \times B)F_1$	A C	++

* General design of experiments conducted to determine whether 4- to 10-day-old F_1 hybrids can discriminate effectively between immunologically competent cells of parental origin (A) and cells of unrelated origin (C). Thus far in experiments this has been the case only when C belonged to an H-2 group different from the H-2 groups of A and B.

TABLE 2

GRAFT-VERSUS-HOST ASSAY OF "FETAL CELL" CHIMERA

C3H × ST/A spleen index	Grafted spleen cells	C3H × DBA/2 spleen index
1.13 1.17	Chimera; cells 2×10^7 each	2.03 3.30
1.65 1.68	C3H (positive control); cells 1×10^7 each	2.03

Let me illustrate this by a concrete example. Fetal C3H liver cells are injected I.P. into an infant hybrid of C3H and ST/A with the purpose of forming a chimera consisting of hybrid cells genetically tolerant of the graft and of grafted cells with acquired tolerance of the ST/A component of the host. When due time has been allowed for immunological maturation of the grafted cells, the presumably chimerical spleen is removed and made into a cellular suspension. A part of the suspension is then tested in (C3H × ST/A) hybrids and another part in (C3H × DBA/2) hybrids, the latter being 4 to 10 days old. If tolerant C3H cells in fact are present in significant amounts, they should reveal their presence by causing splenomegaly in the hybrid offspring of DBA/2 but not in those of ST/A. The best kind of negative control in either case will be the injection into littermates of normal (C3H × ST/A) cells. Positive controls, of course, in either case must be injected with normal adult C3H spleen cells. In the following experiment of this kind, chimera formation was attempted by the grafting of 3.5 × 10^7 fetal C3H liver cells into 2-day (C3H × ST/A) F_1 hybrids, and the animals were analyzed 52 days later (TABLE 2).

The conclusion to be reached from this experiment is that the supposed chimera was in fact a chimera and that the donor component of its spleen had lost reactivity to ST/A antigens and had retained reactivity to DBA/2. In short, the graft had acquired immunological tolerance of its host.

TABLE 3

ADULT SPLEEN CELLS 2 × 10^7 C3H INJECTED INTO 1-DAY-OLD (C3H × ST/A) IN ANIMALS SACRIFICED 19 DAYS LATER

	Weight		
	Body (gm.)	Spleen (mg.)	Liver (mg.)
Injected	6.83	38	522
	7.30	38	603
Not injected	9.63	61	595
	9.38	68	553

TABLE 4

GRAFT-VERSUS-HOST ASSAY OF "ADULT CELL" CHIMERA

C3H × ST/A spleen index	Grafted spleen cells	C3H × DBA/2 spleen index
1.14 1.10	Chimera; 10^7 cells each	1.85 1.83 2.42
2.28	C3H (positive control); 10^7 cells each	1.80 2.55 3.00

Let us now consider another experiment that is indicative, I think, of acquired tolerance in adult spleen cells. It follows the pattern of the previous experiment except that chimera formation is achieved by the grafting of adult spleen rather than of fetal liver.

The injection of adult C3H spleen cells into infant hybrids of the same strain × ST/A of course is to invite runting, which occurred, as shown in TABLE 3. In the present experiment, in which 2 × 10^7 adult spleen cells were injected into 1-day old hybrids, the latter were sacrificed for analysis 19 days later, when they displayed the following signs of runt disease: they were smaller than uninjected littermates, and their spleens were even percentually diminished in size. GVHA made with a pooled suspension of the 2 atrophic spleens gave the results shown in TABLE 4.

Consequently, also in the runting chimera produced by adult parental cells injected into an F_1 hybrid a donor cell population may be found that meets the same requirements of tolerance as did the "fetal cell" chimera: it has lost reactivity to ST/A and retained reactivity to DBA/2 (thus far, chimeras of this kind always have failed to react in (C3H × ST/A). The positive reaction in (C3H × DBA/2), on the other hand, has not been quite consistent, probably depending on the amount of surviving graft cells.

This fact may appear provocatively absurd. Are the hybrids not "runts," since the grafted cells react against the ST/A component and, if they are, how can it be that the grafted cells are tolerant of ST/A?

May I suggest that some reconsideration be given our conception of runt disease in its relation to tolerance and homograft immunity. As a first step, which can be no more than tentative and preliminary, perhaps two kinds of sensitization should be distinguished: productive sensitization and exhaustive sensitization. With a given combination of strains, these two modes of reaction may be demonstrated in the reciprocal experimental situations schematically outlined in TABLE 5. Whereas the arrangement recommended for the demonstration of productive sensitization implies that GVHA is made on host cells (A) with prior experience of a foreign graft that cannot react on the host, exhaustive sensitization involves a similar GVHA on graft cells (A) with the prior experience of a host that cannot react on the graft. In either case the antigenic stimulation is qualitatively the same. However, it is understood that, during preactivation, in the former case the stimulated A cells are confronted with a limited and diminishing amount of antigen while, in the latter case, they are constantly exposed to an excess of the antigen (a concrete example of productive sensitization, outlined in TABLE 5, may be found in Simonsen and Jensen, 1959 section on gene dose effect). TABLE 4 of the present paper represents exhaustive sensitization.

TABLE 5
SENSITIZATION

Adult
(A × B)→A : Productive sensitization
⋮
Sensitized A ↘ Infant (A × B) } More splenomegaly with sensitized A than with normal A
Adult normal A ↗

Adult A Infant (A × B) : Exhaustive sensitization
⋮
Sensitized A ↘ Infant (A × B) } No splenomegaly with sensitized A, although they may react in (A × C)
Adult normal A ↗

Productive sensitization probably needs little further comment here, since it is the conventional form of homograft sensitization that leads to an increased number of immunologically competent cells with specific affinity to the graft antigens. Although I do not pretend to know what it really is, the following concept of exhaustive sensitization appears to me a reasonable working hypothesis.

When immunologically competent cells are suddenly exposed to a great and persistent overload of homologous antigen, initially they will try to cope with that antigen in the usual fashion, that is, in the pattern of productive sensitization. Since this initial immune response necessarily leads to a situation in which the stimulated cells must develop a further response in great excess of antigen, it seems unavoidable that antigen-antibody reactions take place in or upon the reacting cells of the graft. Thus, quite independently of any host reaction against the graft, those graft cells that have initiated an immune response to the host antigens may well experience some damage by their own reactivity.

Which consequences for the graft cell population are, then, to be expected? I think this will depend on the strength of the antigenic stimulus they receive and also on the actual homogeneity of the population. Let us try to consider the two factors independently. It perhaps is reasonable to expect that the stronger the antigenic stimulus, the more severe also is the damage inflicted on the reacting cells. However, the possibilities range from suicide before the cells reach their first mitotic division to a long-drawn-out impairment of viability and function taking several cell cycles and ending in immunological inertia. Gorer and Boyse (1959) have described experiments in which grafting of parental spleen cells to F_1 hybrids was followed by a rapid disappearance of the graft cells, a fact they have interpreted as allergic death of the graft.

Whatever the strength of an antigenic stimulus, the question of the homogeneity of cell population will have a bearing on the situation. If all immunologically competent cells of the graft have the same potentialities they also should suffer the same damage. If, on the other hand, there exists such a thing as genetic variation in these cells because of somatic mutation (Lederberg, 1959; Burnet, 1959), only some cells should suffer, while others that have no inherent affinity to the host antigens sooner or later should enjoy a selection advantage; hence a shift in population that should lead to something indistinguishable from acquired tolerance should occur.

A very similar result, however, might be expected on instructivist grounds (as Burnet and Fenner, 1949, might have expected), if it is assumed that adult spleens do in fact retain a complement of immature cells that can acquire tolerance actively in the same adaptive way as embryonic cells, according to this theory, are supposed to do. Hence a selection pressure would be exerted that would be to the disadvantage of all immunologically competent cells because, in accordance with this theory, such cells are all virtually alike in their genetic endowment. The selection pressure would therefore favor the immature cells; these would develop tolerance instead of becoming sensitized.

In conclusion, it seems that exhaustive sensitization accompanied by the creation of a tolerant cell population is compatible with both selectionist and instructivist ideas of tolerance, but it may not continue to be so. For example, may I postulate that, since the antigenic difference between two inbred strains cannot possibly cover the whole of the immunological universe, apparently tolerant cells in any strain combination should be left behind if the mutation-selection theory is correct. On the other hand, if the adaptational view is correct, tolerance would develop only under the conditions of exhaustive sensitization in such strain combinations where tolerance is readily induced by conventional means because, according to this theory, tolerance may be achieved more easily the more chemically related are the foreign antigens to some autologous antigens of the host (Simonsen, 1956).

Quite apart from the controversy between selectionist and instructivist ideas, studies of exhaustive sensitization make me increasingly ready to believe that the essential damage inflicted on the host by grafting of immunologically competent cells may occur very early, indeed, since the grafted cell population quickly becomes exhausted of its specific reactivity to the host, although it need not lose reactivity in general. The attempts by Siskind and Thomas (1959) to prevent runting by the adoptive transfer of adult host strain cells point in the same direction, although an extension of their approach to the use of preimmunized donors will be necessary before any firm conclusions may be drawn.

Finally, may I stress the importance for clinical homografting that the study of these problems is likely to have—particularly in respect to overcoming immunological crises under conditions in which the antigenic differences between donor and host are not too great.

One is reminded of two recent, spectacularly successful kidney transplantations done in Boston, Mass., and in Paris, France, where, at least in the French case, it is claimed that the grafted kidney, about 3 weeks after transplantation, went through an immunological crisis that was spontaneously overcome. It seems to me that, if this is true, it might well be a clinical counterpart of the fact that splenomegaly in F_1 hybrids grafted with parental spleen cells reaches a peak after 8 to 12 days and then fades off (Simonsen and Jensen, 1959), as do the other signs of runt disease when the strain combination is not a very dangerous one. It appears to me likely that, under such conditions, in which a host is genetically tolerant of a graft, the spontaneous cure of runt disease is due to exhaustive sensitization* and possibly is accompanied simultaneously by the development of a tolerant cell population.

References

1. Billingham, R. E. & L. Brent. 1959. Phil. Trans. Roy. Soc. London. **B242:** 439.
2. Burnet, M. F. & F. Fenner. 1949. The Production of Antibodies. MacMillan. Melbourne, Australia.
3. Burnet, F. M. 1959. The Clonal Selection Theory of Acquired Immunity. Cambridge Univ. Press. London, England.
4. Gorer, P. A. & E. A. Boyse. 1959. *In* Biological Problems of Grafting. F. Albert & P. B. Medawar, Eds. Blackwell Sci. Publ. Oxford, England.
5. Hamburger, J., J. Vaysse, J. Crosnier, M. Tubiani, C. M. Lalanne, B. Antoine, J. Auvert, J.-P. Soulier, J. Dormont, C. Salmont, M. Maisonnet & J. L. Amiel. 1959. Presse méd. **67:** 1771.
6. Lederberg, J. 1959. Science. **129:** 1649.
7. Simonsen, M. 1956. Acta Pathol. Microbiol. Scand. **39:** 21.
8. Simonsen, M., J. Engelbreth-Holm, E. Jensen & H. Poulsen. 1958. Ann. N. Y. Acad. Sci. **73**(3): 834.
9. Simonsen, M. & E. Jensen. 1959. *In* Biological Problems of Grafting. F. Albert & P. B. Medawar, Eds. Blackwell Sci. Publ. Oxford, England.
10. Siskind, G. W. & L. Thomas. 1959. *Ibid.*

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* The term exhaustive sensitization has been used throughout instead of immunological paralysis because, to my knowledge, there is no indication that the latter is preceded by intense proliferation of immunologically competent cells.

Discussion of the Paper

Myron S. Silverman (*United States Naval Radiological Defense Laboratory, Berkeley, Calif.*): For the past few years we have been interested in the induction of tolerance to rat cells in lethally irradiated mice protected by rat bone marrow transplants where, as you know, secondary disease occurs in a large percentage of the mice so treated. We have been interested primarily in studying the immunological competence of the two components of the chimera, the rat cells and the host-mouse cells. We have made the following observations in a series of experiments that I shall list very briefly: (1) the inability to transfer antibody formation to the irradiated mice from immune rats by rat bone marrow (although we can do this with spleen cells obtained from the same rats); (2) the inability to induce any immune response in mouse chimeras by the injection of tetanus toxoid or sheep red blood cells or similar antigens; (3) the inability to accelerate the death or secondary disease by using bone marrow from rats that have been immunized against our recipient strain of mice; and (4) the finding that our rat bone marrow chimeras not only did not respond to the injection of tetanus toxoid or sheep red blood cells in an immunological sense, but that a good proportion of the few that we have tested have accepted skin grafts not only from the strain of rat that contributed the marrow protecting them, but also from strains of rats histoincompatible to the donor rats. Furthermore, these chimeras would accept skin grafts from the strain of mouse that is histoincompatible with our mouse strain, indicating, therefore, a complete incompetence in so far as the immune response is concerned.

We are still faced, however, with the fact that these animals are dying from secondary disease. It has occurred to us recently that, if one accepts the Burnet-Lederberg-Jerne selective theory of antibody formation, one can postulate that the bone marrow cells that we are injecting and that obviously have a small number, comparatively speaking, of antibody-forming cells are met with a massive amount of mouse antigen. Therefore, it could very well be that perhaps the only rat antibody-forming cells that can survive and repopulate the mouse and then continue to react against the mouse tissues are those that through their peculiar mechanism can form antibodies only against the mouse. If, then, that were truly the case, we are faced with the terrible fact that no matter what we do immunologically we shall never be able to prove whether these animals have cells producing antibodies against the host because the systems that we use are those that try to show whether or not these host animals are immunologically competent. These animals will not form antibodies against themselves, obviously, yet the only cells we have left to consider are those that are forming antibodies against the host. Therefore, we have no way of demonstrating, except through pathologic examination, the fact that an antibody process on the part of the donor cells is taking place. Perhaps at the next conference in 1962, I shall be able to explain this a little more thoroughly; at that time we may have a roundabout method of proving whether this hypothesis is correct.

J. V. Nossal: I remark on what a continuing stimulus the work of Simonsen has been to all of us interested in theories of antibody formation.

I mention two studies completely inspired in every aspect by Simonsen's original work in the chick embryo and bearing considerable relevance to the paper today.

The first study is by Boyer and Burnet, and I believe it appeared in *Nature*. It concerns the transplantation of adult fowl leukocytes onto the chorioallantoic membrane of developing chick embryos. If you choose the age of the developing chick embryo rather correctly and rather carefully, using chick embryos somewhat younger than were used in the early studies of Simonsen, these adult fowl leukocytes, or at least a certain proportion of them, can proliferate and actually form marked red clans or little nodules of fifty or one hundred mononuclear cells each, which appear to proliferate and can then be passaged, to a certain extent, from one immunologically neutral host chick embryo to the next.

I think this type of study, which is being actively pursued by Burnet now is the most promising lead that we have to a pure clan technique in immunology, one that eventually may prove or disprove the Jerne-Burnet-Lederberg concepts.

The second study is one of our own, also inspired entirely by the Simonsen spleen transfer experiments, but more similar to those he describes in his paper. Its purpose is to find out, having demonstrated the immunological competence of the population of lymph node or spleen cells, whether these cells maintain or do not maintain that competence on serial transfer. Using the system of adult spleen cells transplanted into baby isologous mice, we have been able, in this fashion, harvesting the spleen of the host mass about three or four weeks later and using a Harris-type *in vitro* stimulation of the spleen cells at each serial passage, to maintain antibody production in a population of spleen cells for a period of eight months. It appears that the only type of antibody-producing capacity that persists under such circumstances is that which the original adult spleen cell population possessed. In other words, if the original spleen cell population is producing only antibody *A* and *B*, one can obtain continued production of both *A* and *B*, or of *A*, or of *B*, under appropriate circumstances, depending upon how one sets up the experiment, but it will never be possible to maintain antibody production against antigens *C* or *D* or anything else.

In this type of situation the Simonsen technique also appears to be the only one of really direct relevance to theories of antibody formation.

SIMONSEN: I have very few comments to make. I am very interested in Silverman's mention of the fact that the mouse-rat irradiation chimera would accept rat skin of strains other than the one used as a donor strain. This, I think, fits very well with the experience of Hašek and his group in Prague, Czechoslovakia, who did not work with irradiated animals but with embryonic parabiosis of avian eggs and, in so far as I remember, in some cases managed to induce tolerance by this means between chicken and turkey, for example. Hašek and his colleagues found that this tolerance sometimes extended to turkeys other than the one used for parabiosis, and similar findings are true also for the heterologous blood.

I think also that Billingham and Brent, in their recent publication, have some evidence that acquired tolerance in mouse strains is not always as specific as we formerly thought.

KOPROWSKI: Boyer and Burnet's investigations, mentioned by Nossal, represent an extension of Burnet's technique of virus-counting to the field of immunology. Burnet has used the chorioallantoic membrane for counting virus particles for the past twenty-five years, and I think he merely substituted fowl leukocytes and obtained similar and very exciting results and, probably, a technique that may be applicable to many fields of immunology.

IMMUNE HEMOLYSIS: A FEATURE OF SECONDARY DISEASE AND RUNT DISEASE IN THE RABBIT*

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When rabbit fetuses are injected with homologous spleen cells from an adult animal, about 1 week after the injection a considerable proportion of the recipients stop growing, become wasted, and develop anemia.[1] Usually they soon die, but sometimes, stunted, they struggle on, suffering from a process that has come to be called runt disease[2]. This condition does not occur if the suspension used for injection is either lacking in antibody-producing cells or consists solely of embryonic cells.

A phenomenon essentially similar to runt disease may account for the late deaths that often occur after treatment of lethally X-irradiated rabbits with bone marrow from other normal rabbits.[3] The first effect of the injected cells is to protect these otherwise doomed animals by repopulation of their depleted marrow spaces, leading to rapid hematopoietic recovery and prevention of death from infection or hemorrhage. After this initial recovery some of the animals later develop wasting, diarrhea, a leukocytosis and, often, anemia and jaundice. The outcome of this so-called secondary disease is variable; in many animals it is lethal, but a few survive and gradually regain a normal appearance. Secondary disease can be avoided if early fetal hematopoietic tissue instead of adult bone marrow[4] is used in the treatment of the irradiated animals.

The pathological changes in the two syndromes are similar. In both instances, by using sex chromatin as a biological marker, it has been shown that the injected foreign cells or their descendants persist and are present in the lymph nodes and spleen[5]. These animals are therefore chimeras. At first, free proliferation repopulates the lymphoid system of each with pyronin-positive donor cells resembling the transitional and immature plasma cells described by Fagraeus.[6] During this phase the spleen is enlarged. After the initial increase there is eventual regression, with atrophy of the new tissue and its gradual replacement by collagen and scattered collections of mature plasma cells. Focal liver necroses are also a feature of both conditions. The only real difference is seen in the bone marrow. In secondary disease the restored marrow is always cellular, whereas in runt disease it is frequently hypoplastic or aplastic.[5]

It is thought by many that runt disease and secondary disease are immunological illnesses and, more particularly, illnesses caused by reaction of the grafted cells against the host.[7] Further support for this concept is given in this paper, and evidence is presented that an immune hemolysis of host red cells is a feature of both conditions.

Materials and Methods

Chinchilla rabbits that were not inbred in the genetic sense were used throughout this study.

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Production of Radiation Chimeras

Lethally X-irradiated rabbits were treated intravenously with bone marrow cells from normal adult rabbits.

X irradiation. Whole-body X irradiation was given as a horizontal beam to 2 animals at a time from a Westinghouse machine under the following conditions: 220kV., 12.5mAmp., 70 cm. target-to-skin distance, 1.0 mm. Cu and 1.0 mm. Al filters, HVL 1.8 mm. Cu, dose rate to skin 33.4 r/min., to center of animal 21.1 r/min. The dosage was divided: an initial dose of 600 r at the center of the animal was followed 24 hours later by 500 r, and after another 24 hours by a further 500 r, giving a total of 1600 r (LD_{100}/14 days).

Adult bone marrow and fetal hematopoietic tissue. Sterile suspensions of 20-day fetal rabbit liver and adult female marrow were prepared as described elsewhere[3,4] and injected into the marginal ear vein of the recipient male rabbit 1 to 3 hours following the last dose of irradiation.

Criterion for success. The successful establishment of a radiation chimera was accepted only if female-type heterophils (amphophils) appeared in the irradiated male rabbit.[8]

Induction of Immunological Tolerance

Spleen cells from adult homologous female rabbits were injected into fetal rabbits.[1]

Preparation of spleen cell suspensions. A mature female rabbit was anesthetized, and a splenectomy was performed through an upper midline abdominal incision. Immediately after removal, the spleen was chopped finely under aseptic conditions in heparinized saline solution and then suspended by gently drawing up and down through a wide-bore cannula. After filtration through nylon bolting cloth of 90-μ porosity a cell count was made, and a suitable quantity of the spleen suspension containing 50×10^6 nucleated cells was injected into the recipient animal.

Injection into fetal rabbits. A rabbit at the twentieth or twenty-second day of pregnancy (the normal gestation period is 31 days), was anesthetized, and a midline abdominal incision was made extending from the level of the last pair of nipples to just below the umbilicus. When the abdomen had been explored to determine the number of fetuses present, the free end of 1 horn of the uterus was delivered through the wound. The position of each fetus was easily seen and its head and back identified by palpation. Adult spleen tissue suspension was then injected through the wall of the uterus into the peritoneal cavity of each fetus. After returning the uterus to the abdomen by gentle steady pressure, the peritoneum and linea alba were sutured as 1 layer with 3-0 chromic gut. The skin was sutured separately.

Weight. The animals were weighed daily.

Antibiotic therapy. All irradiated rabbits were given tetracycline hydrochloride intramuscularly 50 mg./day for the first 2 weeks following irradiation.

Radiochrome tagging. Red cell survival was measured with Cr^{51}-labeled cells. The radiochrome used was a sterile saline solution of radioactive sodium chromate ($Na_2Cr^{51}O_4$) with a specific activity of 10 mc./mg. chromium.

For adult animals 5 μc. of radioactivity and not more than 4 μg. of chromium metal were added to each milliliter of blood treated. Four ml. of blood was drawn from the marginal ear vein immediately after irradiation and before marrow injection, heparinized and centrifuged, and the red cells mixed with the correct volume of radiochrome solution. After being gently agitated at room temperature for 30 min. the cells were washed 3 times in plasma-saline and finally suspended in saline to a volume of approximately 4 ml.; 3 ml. of this suspension was injected. Samples of 0.3 ml. were taken at 1 hour, at 24 hours, and at appropriate times thereafter. Each sample was placed in a small tube containing 4 ml. of saline and lysed in the deep-freeze apparatus.

Newborn rabbits were injected with 0.3 ml. of blood labeled with $Na_2Cr^{51}O_4$ at concentrations of 50 μc./ml. of red cells. Samples of 0.02 ml. were then taken from each rabbit at 1 hour, at 24 hours, and at appropriate times thereafter, the erythrocytes were lysed in 1 ml. of distilled water, and the radioactivity was determined.

Counting was carried out in a well-type scintillation counter, using a thallium-activated sodium iodide crystal. The 1-hour sample was taken as 100 per cent, and the radioactivity of subsequent samples was expressed as a percentage of this value.

Antiglobulin reaction. An adaptation of the antiglobulin reaction of Coombs *et al.*[9] was used. A suitable antirabbit globulin serum was made by immunizing guinea pigs with alum-precipitated rabbit globulin, following the methods used by Kellner and Hedal.[10] The serum was inactivated at 56° C. for 30 min. and absorbed 2 or 3 times with equal parts of packed normal rabbit red cells. Only those sera were used for the Coombs' test that, after absorption, agglutinated rabbit cells coated with a potent anti-F serum in a dilution of 1/128 or higher and did not agglutinate normal rabbit cells. The Coombs' serum was preserved at −20° C. in 2.0-ml. volumes.

Quantitative direct Coombs' test. This was performed in a hot room at 37° C. rather than at room temperature because of the presence of nonspecific cold agglutinins in normal rabbit sera. Serial fourfold dilutions of the antiglobulin serum were made, and a drop of saline served as a control. Next, 1 drop of a 10 per cent suspension of the thrice-washed red cells under test was added to each dilution on an opalescent tile. Normal unsensitized corpuscles and red cells previously sensitized in an anti-F serum were suspended in the antiglobulin serum alongside the test corpuscles and served as further controls: the former for the specificity and the latter for the sensitivity of the reaction. The suspensions were then mixed, and the results read after 5 to 7 min. and scored from ++++ to ±.

Quantitative indirect Coombs' test. For this host red cells collected before irradiation and subsequently stored were used. One drop of a 30 per cent suspension of these red cells was incubated in a tube for 2 hours at 37° C. with 5 drops of the chimera's serum. At the end of this time the tubes were inspected for agglutination, the cells were then washed in 3 changes of saline, and agglutination tests were carried out as for the direct test described above, using the antirabbit globulin serum in varying dilutions.

Elution of antibody from the red cells was attempted, using the method described by Cohen;[11] bilirubin estimations were carried out, using the method of Malloy and Evelyn.[12]

Hematology. Total and differential leukocyte counts, reticulocyte and platelet counts, and microhematocrits were determined at regular intervals. Blood grouping was carried out on many of the rabbits, using trypsin-treated cells.

Experimental procedure. In this experiment 5 groups of animals were studied.

Group I consisted of 9 normal adult rabbits and 10 newborn rabbits of various ages. Tests on animals from this group were made at appropriate times for comparison with those performed on rabbits from the treated groups.

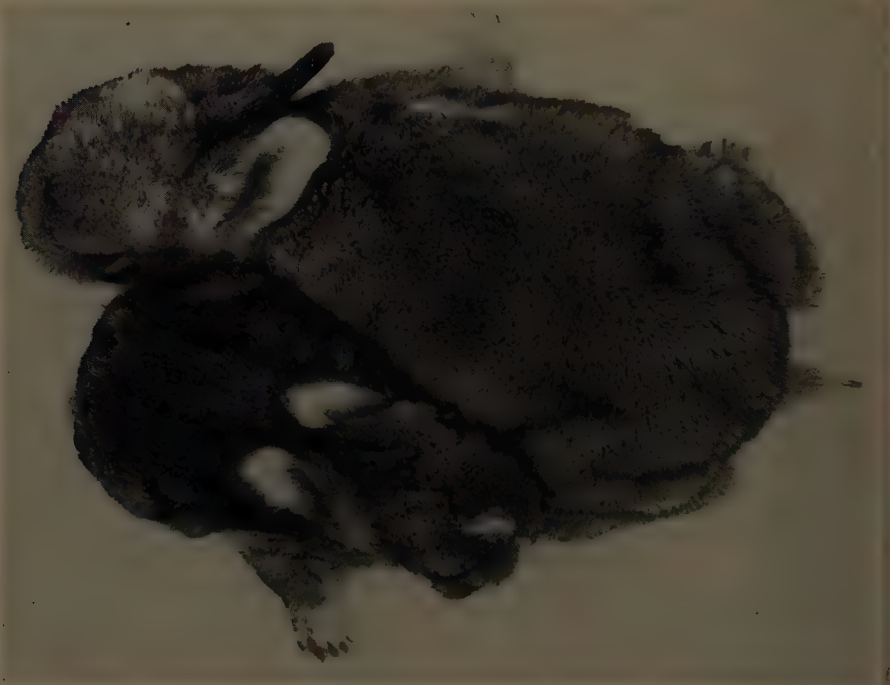

FIGURE 1. Two 14-day-old tolerant rabbits. The smaller of the littermates is suffering from runt disease.

Group II was composed of 9 rabbits that had been exposed to 1600 r whole-body X irradiation, but given no subsequent treatment.

Group III consisted of 6 radiation chimeras restored by 20-day fetal liver.

Group IV consisted of 9 radiation chimeras with secondary disease. In the collection of this group 20 irradiated bucks that became successful chimeras after marrow treatment were studied. Subsequently 9 of these animals developed secondary disease: 6 were from the group given Cr^{51}-labeled host red cells and 3 were from those given tagged erythrocytes from the marrow donor.

Group V consisted of 14 nonirradiated tolerant rabbits with chronic runt disease (FIGURE 1).

In all the animals thought clinically to be suffering from secondary disease runt disease the diagnosis was subsequently confirmed by gross and microscopic post-mortem examination.

RESULTS

Radiation Chimeras

The data for normal and irradiated control rabbits for radiation chimeras produced by fetal tissue, and for chimeras with secondary disease have been published in detail elsewhere.[13] An outline of these findings is given here for comparison with those to be described in runt disease.

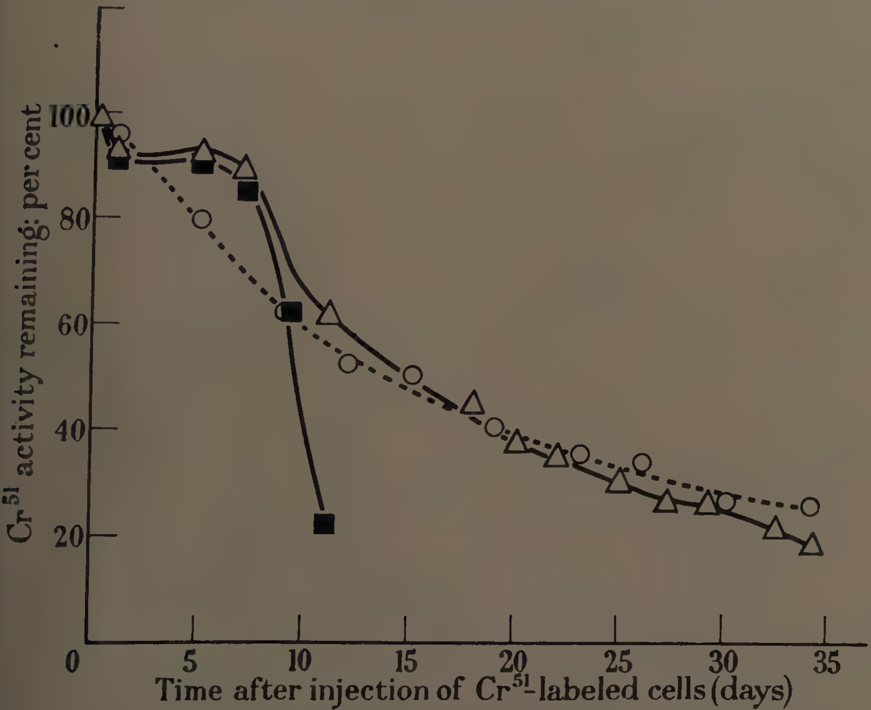

FIGURE 2. Survival of Cr^{51}-labeled autologous red cells in normal and lethally irradiated rabbits and of labeled host erythrocytes in a radiation-chimera produced by fetal liver. Key: ○------○, normal nonirradiated rabbits (mean of 6); ■——■, irradiated control; △——△, irradiated rabbit treated with fetal tissue.

In all of the irradiated rabbits (Groups II, III, and IV) there was a rapid loss of Cr^{51} in the first few days compared with the loss in normal rabbits (FIGURE 2). This phenomenon has been noted previously in irradiated dogs and is poorly understood.[14]

Between the fourth and sixth days in the irradiated animals the Cr^{51} count fell only slightly because of cessation of red cell production.

In the irradiated control rabbits (Group II) a second rapid fall in the Cr^{51} count occurred on the seventh day, due mainly to extravasation of red cells into the tissue and lymph spaces and hemorrhage into the gut. These effects were associated with capillary damage and thrombocytopenia and culminated in the death of the animal.

In rabbits treated with fetal liver (Group III), this second loss of Cr^{51} activity was neither so precipitous nor so prolonged as in the irradiated controls, and it coincided with a rapid rise in the reticulocyte count. In these cases an important factor was probably dilution of the Cr^{51}-labeled cells by an outpouring of newly formed untagged cells from the marrow transplant. By the twelfth day the Cr^{51} loss had slowed, and thereafter the slope of the survival curve of those animals treated with fetal tissue was similar to that obtained for normal rabbits and reflected normal senescence.

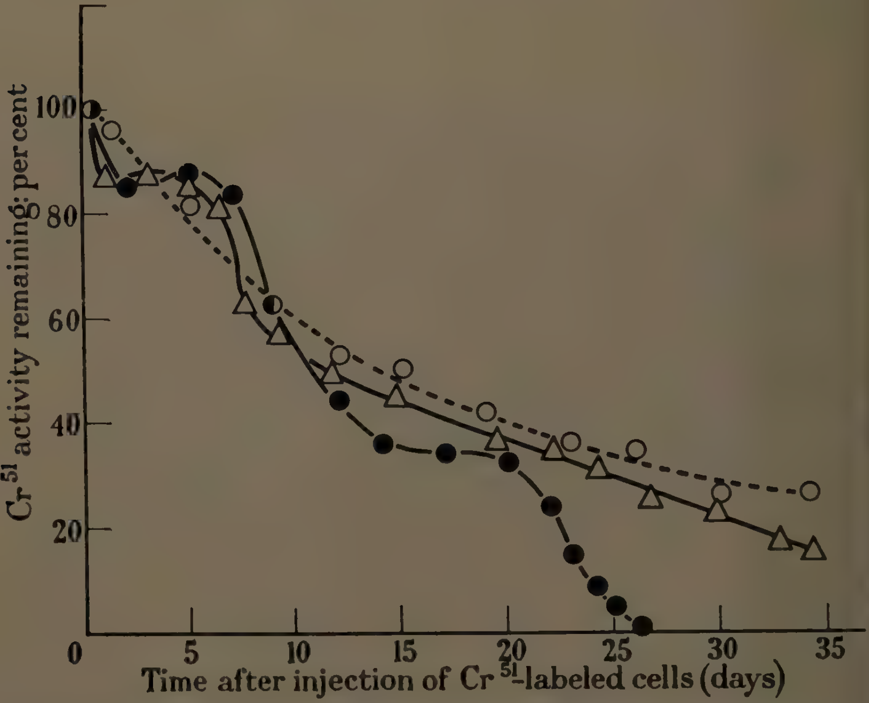

FIGURE 3. Survival curves for Cr^{51}-labeled donor and host red cells in a radiation chimera suffering from secondary disease. The curve for normal rabbits is included for comparison. Key: ○------○, normal nonirradiated rabbits (mean of 6); △——△, radiation chimera in which secondary disease developed (donor cells labeled); ●——●, radiation chimera with secondary disease (host cells labeled). Note the hemolysis commencing at the twentieth day.

The advent of secondary disease in the 6 radiation chimeras of Group IV with labeled host red cells was associated with a third abrupt fall of the Cr^{51} count, which this time continued to zero (FIGURE 3). This third fall was accompanied by a reticulocytosis, spherocytosis, drop in packed cell volume (*see* TABLE 1), increased indirect serum bilirubin concentration, and splenomegaly with splenic hemosiderosis and erythrophagocytosis, all of which favor the loss of chromium caused by the destruction of red cells. Moreover, the hemolysis was selective in that only host erythrocytes were destroyed; transfused labeled donor cells were unaffected.

Sensitized cells were sought as soon as the hematocrit plasma became tinged with yellow, and a positive direct Coombs' test was obtained in each of the radiation chimeras with secondary disease. The indirect Coombs' test was negative in 3 animals and positive in low dilution in 3 others. Further investigations have since been carried out on material from these animals (TABLE 2), and attempts to test the specificity of the coating substance performed in all cases with a positive direct Coombs' test. In 3 instances eluates from the host's red cells have showed an antibody that is specific for red cells of the host's blood group.

TABLE 1

TERMINAL HEMATOLOGICAL FINDINGS IN CHIMERAS (MEAN VALUES)

Group	Bone marrow at post-mortem	No. of rabbits	P.C.V.	Reticulocytes	Nucleated red cells (per 1000 red cells)	Heterophils (per mm.^{3})	Lymphocytes (per mm.^{3})	Platelets ($\times 10^3$/mm.^{3})
Normal baby rabbits (4–9 weeks)	Normal	9	40	12.1	2.3	1,800	1,670	550
Runts	General aplasia	3	14.1	0	0	950	375	100
	General hypoplasia	5	25.7	2.0	0	1,100	420	185
	Normal	2	34.2	16.3	3.4	2,500	600	480
	Myeloid hyperplasia	4	30.3	14.8	2.2	5,000	520	510
Normal adult rabbits	Normal	9	38.6	2.0	0.6	2,400	4,400	750
Radiation chimeras with secondary disease	Myeloid hyperplasia	9	21.6	14.2	1.8	10,800	600	800

TABLE 2

DILUTIONS OF ANTIGLOBULIN SERUM GIVING MAXIMUM AGGLUTINATION OF RED CELLS IN RABBIT RADIATION CHIMERAS SUFFERING FROM SECONDARY DISEASE

Animal	Blood type*		Coombs' test		Specificity of eluate
	Donor	Recipient	Direct	Indirect	
1	F	A	1/4	—	—
2	F	A	1/64	Not done	—
3	F	A	1/4	—	—
4	A	F	1/64	1/8	anti-F
5	F	A	1/16	Not done	—
6	F	A	1/16	Not done	—
7	A	F	1/64	1/32	anti-F
8	F	A	1/4	—	—
9	F	A	1/256	1/64	anti-A

* Nomenclature according to Cohen.[15]

Runt Disease

Red Cell Survival Studies

Normal baby rabbits (*Group I*). Nine normal baby rabbits varying in age between 3 and 9 weeks were injected with a sample of their own red cells tagged with Cr^{51}; the curve of survival is shown FIGURE 4. The mean 50 per cent survival time of these cells was 10.4 ± 4.3 days, compared with 15 ± 2.3 days for normal adult rabbits.

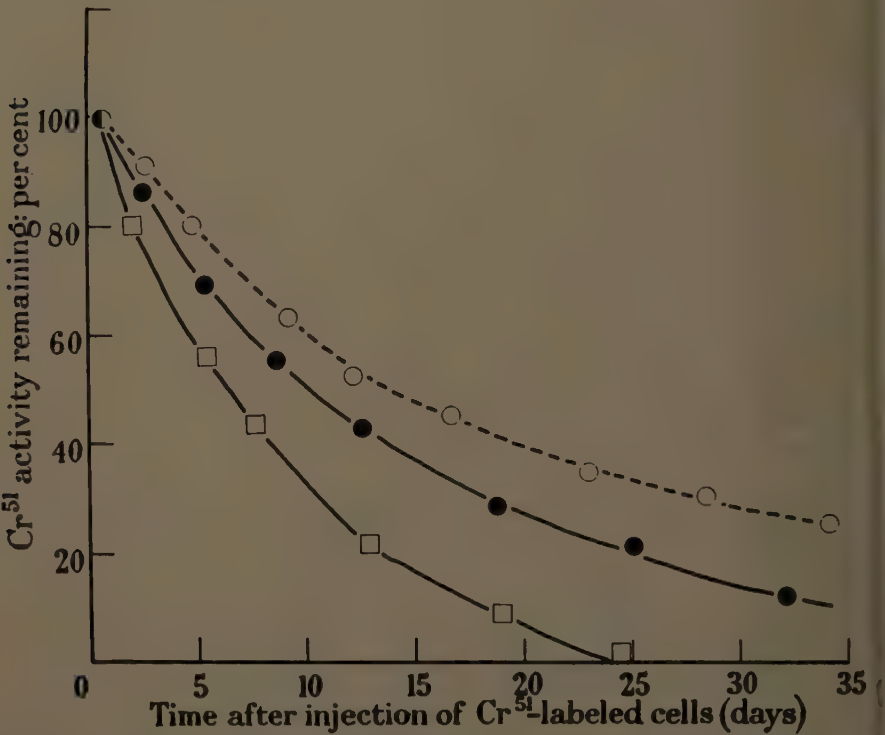

FIGURE 4. Survival curves for Cr^{51}-labeled host and spleen-donor red cells in tolerant rabbits suffering from runt disease. The curve for tagged autologous red cells in normal baby rabbits is included for comparison. Key: ○------○, tolerant rabbits suffering from runt disease (spleen-donor cells labeled—mean of 3); ●——●, normal baby rabbits aged 4 to 9 weeks (mean of 9); □——□, tolerant rabbits suffering from runt disease (own cells labeled—mean of 6).

Tolerant rabbits with runt disease (*Group V*). Nine such rabbits were studied. In 6 animals a sample of their own blood was labeled and reinjected; in the remaining 3 rabbits homologous adult blood from the spleen donor was tagged and given.

All 3 of the latter animals showed curves of disappearance of Cr^{51} from the circulation (FIGURE 4) closely similar to those found in normal adult rabbits given labeled autologous cells. The mean 50 per cent survival time of these cells was 14.7 ± 3.1 days.

In the runts that were given their own labeled red cells these bodies disappeared more rapidly than the autologous cells of a normal baby rabbit and much more rapidly than labeled adult donor cells. The mean 50 per cent survival time of the chimeras cells was 6.4 ± 1.9 days.

Hematology

All the newborn rabbits showed the normal variations in hematological values noted previously by other workers.[16] Even at this stage, however, some of the animals, which were subsequently shown to be suffering from runt disease, tended to have lower average levels of all blood elements except lymphocytes, which were generally in the normal range. As the disease progressed, 8 of the runts showed a steady fall in numbers of all the blood cells (TABLE 1) and, at death, the bone marrow was found to be aplastic or hypoplastic. The other 6 runts, after an initial fall, showed, 2 to 3 weeks after birth, a gradual increase in the packed cell volume, reticulocytes, nucleated red cells, and granulocytes; in some a heterophil leukocytosis occurred terminally. The lymphocyte count did not follow this trend and fell steadily from about the second week until death. At necropsy the bone marrow was normal or hyperplastic. Spherocytes were usually present at some stage in the blood of all the affected animals and were sometimes numerous. In the runts with normal or hyperplastic bone marrow, the association of spherocytes and reticulocytes resulted in pronounced anisocytosis and polychromasia.

TABLE 3

DILUTIONS OF ANTIGLOBULIN GIVING MAXIMUM AGGLUTINATION OF RED CELLS AND SERUM BILIRUBIN LEVELS IN TOLERANT BABY RABBITS SUFFERING FROM RUNT DISEASE

Animal	Blood type*		Direct Coombs' test	Specificity of eluate	Serum bilirubin (mg. per cent)	
	Spleen donor	Runt			Direct	Indirect
1	F	A	1/64	—	0.1	1.6
2	A	F	1/256	anti-F	0.4	0.8
3	F	AFJ	1/256	anti-A	0.3	0.9
4	A	A	—	—	0.1	0.2
5	F	A	1/64	—	0.4	1.2
Normal neonatal rabbits	—	—	—	—	0.1	0.3

* Nomenclature according to Cohen.[15]

Serology

Five of the animals suffering from runt disease were examined serologically and found not to have any demonstrable complete in-saline agglutinating antibody in their serum, but the red cells of 4 others gave a positive direct antiglobulin reaction (TABLE 3). No true prozone of inhibition due to antibody excess was encountered, although agglutination was better after dilution of the antiserum. Agglutination of the cells was readily inhibited by small amounts of rabbit gamma globulin that suggested that the coating substance was composed principally of gamma globulin. That this coating material was a specific antibody was demonstrated in 2 cases by eluting it from the cells at 56° C. and using the eluate to coat known cells. The indirect Coombs' test was not carried out in this group of runts.

Bilirubin Estimations

Twelve of the 14 runts studied were found to have a raised serum indirect bilirubin, with a mean of 1.2 ± 0.5 mg. per cent, compared with a value of 0.3 ± 0.2 mg. per cent for the control group of normal baby rabbits. The direct bilirubin levels were also sometimes raised, but only slightly. The livers of 6 of the runts at post-mortem showed focal liver necroses. Biliary cirrhosis was not encountered.

DISCUSSION

These findings underline the essential similarity of secondary disease and runt disease in the rabbit. In both, the host is immunologically defenseless; in secondary disease because of irradiation damage and in runt disease because the immune system is insufficiently mature. The injected foreign cells proliferate and invade and repopulate the lymphoid system of the host. It is thought that the transplanted cells then begin to attack the host tissues. The present experiments show that at this stage there certainly is hemolysis of host erythrocytes, apparently due to coating antibody of incomplete "warm"-type specificity for host blood group antigens. This confirms a previous finding[17] that the descendants of the implanted cells are capable of elaborating antibody in their new environment and now extends it further to show that they are also capable of forming isoantibody directed against host antigens. The mechanism of attack on host cells other than erythrocytes, however, is still open to speculation. Furthermore, why histological evidence of such an attack, which one would expect to be widespread, should be confined to hematopoietic, lymphoid and, just possibly, hepatic tissue, is equally obscure.

Moreover, despite the postulated assault, death of the chimera rarely occurs at this time, but is usually postponed until the destruction of the new lymphoid tissue leaves the animal bereft of both host and donor antibody-producing cells. Massive infection then quickly supervenes, and the immunological cripple succumbs.

At present this secondary loss of donor cells cannot be explained satisfactorily. That it is not due to a reaction by resurgent or newly intolerant host lymphoid cells is shown by its occurrence in F_1 hybrid mice invaded by parental spleen cells where we know the host is incapable of evicting the transplanted cells.[18] Kaplan and Rosston[19] suggest that the foreign cells probably die in the course of killing host target cells, a process that, these authors consider, might necessitate direct contact between each donor cell and a very few target cells. Gorer[20] has postulated that the foreign lymphoid tissue does not survive because it is susceptible to allergic destruction. He considers that antibody may be fixed to the grafted cells and, when this reacts with the excess of antigen in the environment, cytolysis follows. It may even be that the excessive amount of host antigenic material coming to the foreign lymphoid tissue simply overwhelms and exhausts it. At present we have insufficient evidence to indicate which, if any, of these hypotheses is correct.

SUMMARY

Rabbit radiation chimeras suffering from secondary disease and tolerant rabbits with runt disease both develop anemia, spherocytosis, and a raised indirect serum bilirubin.

These changes are accompanied by a rapid selective loss of Cr^{51}-labeled host erythrocytes, transfused red cells from the marrow or spleen donor being unaffected. Serologic findings show that both syndromes are associated with the presence of a coating antibody of the incomplete warm type specific for host red corpuscles.

It is concluded, therefore, that an important feature of secondary disease and runt disease is an immune hemolysis of the recipients' erythrocytes caused by antibody produced by the transplanted foreign lymphoid cells.

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REFERENCES

1. Porter, K. A. 1960. Runt disease and tolerance in rabbits. Nature. **185:** 789-790.
2. Billingham, R. E. & L. Brent. 1957. A simple method for inducing tolerance of skin homografts in mice. Transplantation Bull. **4:** 67-71.
3. Porter, K. A. & J. E. Murray. 1958. Long-term study of X-irradiated rabbits with bone marrow homotransplants. J. Natl. Cancer Inst. **20:** 189-205.
4. Porter, K. A. 1959. Use of fetal haemopoietic tissue to prevent late deaths in rabbit radiation-chimaeras. Brit. J. Exptl. Pathol. **40:** 273-280.
5. Porter, K. A. 1960. Graft-versus-host reactions in the rabbit. Brit. J. Cancer. In press.
6. Fagraeus, A. 1948. Antibody production in relation to the development of plasma cells. *In vivo* and *in vitro* experiments. Acta Med. Scand. Suppl. **204.**
7. Trentin, J. J. 1958. Tolerance and homologous disease in irradiated mice protected with homologous bone marrow. Ann. N. Y. Acad. Sci. **73**(3): 799–810.
8. Porter, K. A. 1957. Use of the sex difference in morphology of polymorphonuclear leucocytes to indicate survival of marrow homotransplants. Transplantation Bull. **4:** 129.
9. Coombs, R. R. A., A. E. Mourant & R. R. Race. 1945. A new test for the detection of weak and "incomplete" Rh agglutinins. Brit. J. Exptl. Pathol. **26:** 255–266.
10. Kellner, A. & E. F. Hedal. 1953. Experimental erythroblastosis fetalis in rabbits. I. Characteristics of a pair of allelic blood group factors and their specific immune isoantibodies. J. Exptl. Med. **97:** 33–49.
11. Cohen, C. 1955. Blood group factors in the rabbit. J. Immunol. **74:** 432–438.
12. Malloy, H. T. & K. A. Evelyn. 1937. The determination of bilirubin with the photoelectric colorimeter. J. Biol. Chem. **119:** 481–490.
13. Porter, K. A. 1960. Immune haemolysis in rabbit radiation-chimaeras. Brit. J. Expt. Pathol. **41:** 72-80.
14. Stohlman, F., G. Brecher, M. Schneiderman & E. P. Cronkite. 1957. The haemolytic effect of ioninzing radiations and its relationship to the haemorrhagic phase of radiation injury. Blood. **12:** 1061–1085.
15. Cohen, C. 1958. On blood groups and confusion in the rabbit. Transplantation Bull. **5:** 21–23.

16. Sabin, F. R., F. R. Muller, K. C. Smithburn, R. M. Thomas & L. E. Hummel. 1936. Changes in the bone marrow and blood cells of developing rabbits. J. Exptl. Med. **64:** 97–120.
17. Hobson, D., K. A. Porter & J. L. Whitby. 1959. The immunological response of marrow treated X-irradiated rabbits to bovine gamma-globulin. Brit. J. Exptl. Pathol. **40:** 15–24.
18. Nowell, P. C. & L. J. Cole. 1959. Pathologic changes in old nonirradiated F1 hybrid mice injected with parental-strain spleen cells. Transplantation Bull. **6:** 435–437.
19. Kaplan, H. S. & B. H. Rosston. 1959. Studies on a wasting disease induced in F1 hybrid mice injected with parental strain lymphoid cells. Stanford Med. Bull. **17:** 77–92.
20. Gorer, P. A. & E. A. Boyse. 1959. Some reactions observed with transplanted reticulo-endothelial cells in mice. *In* Biological Problems of Grafting. : 193–206. F. Albert and P. B. Medawar, Eds. Blackwell Sci. Publ. Oxford, England.

ACQUIRED PARTIAL TOLERANCE TO HOMOLOGOUS SKIN GRAFTS IN THE HUMAN INFANT AT BIRTH*

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It is well established already that acquired immunological tolerance to homografts can be evoked experimentally in certain species by the injection of leukocytes into fetal or newborn animals.[1,2] Postnatal induction of tolerance, however, has been accomplished only by leukocyte transfer in those species of animals that already have not passed beyond what appears to be a "critical period" or "adaptive phase" in their immunological maturation.[1] This period varies from species to species,[1,3,4] from strain to strain[5] and, probably, from antigen to antigen,[3] but seldom extends more than a few days beyond birth, as is the case in mice[5] and rats,[6] for transplantation tolerance. The apparently greater developmental maturity of the newborn human, as compared with newborn mice and rats, might suggest that at birth the human infant has already passed beyond this critical period. However, little has been done to date to determine whether there is any critical period in human development for the induction of immunological tolerance, and the exploratory studies of Woodruff,[7] Peer,[8] and Good[9] have not yet excluded the possibility that homotransplantation tolerance might follow leukocyte transfer in the human neonate.

The induction of tolerance in this fashion, however, is frequently followed in animal experiments by inimical effects in the immature host, of which the phenomenon of "runt disease"[2] is probably the best known in mammalian immunology. These two related phenomena (homograft tolerance on the one hand and runt disease on the other) raise important questions in pediatric practice as to the possible advantages or possible risks of leukocyte transfer to the human infant in the neonatal period. Against the possible advantages that tolerance could confer upon the human neonate for the improved surgical handling of certain congenital anomalies must be set the risks of inducing runting or some other related phenomenon. These questions assume more obvious importance when considered in relation to exchange transfusion.

The likelihood of large-scale transfer of viable leukocytes to human neonates has been greatly increased in recent years by currently accepted practices in the management of exchange transfusion, now an established treatment for hyperbilirubinemia of the newborn. The chief factors operating in this direction are the trend toward plastic and siliconized transfusion equipment and an increasing emphasis on the merits of using blood drawn as freshly as possible in preference to blood that has been stored.[10-12] The immunological consequences for infants so treated have not been explored yet in any great detail. Further studies are still required, therefore, to test the immunological reactivity of the newborn human infant and his behavior toward transplants of homologous cells or tissues. Exchange transfusion for jaundice of the newborn

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affords opportunities not yet fully utilized to explore these questions, which are certainly not without relevance to the exchange procedure itself.

In the present study homografting of adult skin has been combined with exchange transfusion in 12 newborn infants with hyperbilirubinemia. The results of this study indicate a profound effect on transplantation immunity following exchange transfusions with fresh but not with stored blood. Some factor present in the former, but not in the latter (presumably leukocytes), rather than the exchange procedure itself, appears to be responsible for this effect, which was not dependent upon the infants being in an "adaptive phase" of immunological immaturity. The observations raise the pertinent question of whether any specific effects of leukocyte transfer can operate beyond the critical period for induction of tolerance in a manner inimical to mature immunological mechanisms.

TABLE 1

FATE OF SKIN HOMOGRAFTS FOLLOWING EXCHANGE TRANSFUSION FOR NEONATAL JAUNDICE

Group	Patient	Skin donor	Fate of graft	
			Acute homograft rejection	Extended* survival
Stored-blood recipients	R.S.	Blood donor	+	−
	I.N.	Blood donor	+	−
	R.G.	Father	+	−
	I.W.	Father	+	−
	K.B.	Mother	±	±
	M.C.	Mother	−	+
Fresh-blood recipients	S.T.	Blood donor	−	+
	J.L.	Blood donor	−	+
	M.D.	Blood donor	−	+
	T.F.	Blood donor	−	+
	W.G.	Blood donor	−	+
	M.McG.	Blood donor	−	+

* Beyond 21 days.

Methods

Six infants were given fresh blood transfusions and a skin graft from the corresponding blood donor. The blood for these exchanges was drawn just prior to use into Fenwal polyvinyl bags so as to preserve as many viable leukocytes as possible.

For control purposes another 6 infants were given blood stored in glass for 48 hours or more and a graft from either the father, the mother, or the donor of the stored blood (TABLE 1).

The grafts were full-thickness homografts of defatted skin measuring 0.5 × 1.5 cm. and placed on equivalent surgically created full-thickness skin defects in close proximity to the umbilicus.

More detailed data concerning the clinical conditions and relations of the donors and hosts, together with the timing and extent of these various procedures, are listed in TABLE 2.

For multiple exchanges in infants in the fresh blood group the first of the serial transfusions was always with fresh blood from the selected skin donor. Except for 1 infant (M.D.) who received 2 successive fresh transfusions from the skin donor, subsequent exchanges in these infants were performed with stored blood from different donors. A separate unrelated donor was used on each occasion for multiple exchanges in the stored blood group.

Except for 2 applications of maternal grafts (K.B. and M.C.), only male skin donors were used.

Except in 2 instances (J.L. and W.G.), all the donor blood used in the exchanges was type O, D negative. The 2 exceptions were in O, D-positive children, who received skin and fresh blood donations from O, D-positive males.

One infant (I.W.) in the control group was born prematurely at an estimated gestational age of 32 weeks and weighed 3 pounds at birth. Two of the infants in the fresh-blood group were considered premature on the basis of their birth weights (J.L., weighing 4 lb., 6 oz., with no reliable information as to gestational age, and T.F., 4 lb., 11 oz. after an estimated gestation of 36 weeks). In these 2 premature infants there was a possibility that maternal-fetal sensitization by an incompatibility of ABO blood types may have been an etiological factor in their jaundice, and this possibility also existed in 3 other infants (M.C., S.T., W.G.) in one of whom (M.C.) both BO and D incompatibility were considered. However, diagnosis of erythroblastosis fetalis (EBF in TABLE 2) was not made in the face of negative Coombs' tests and no other positive evidence of recognized incompatibilities. Hyperbilirubinemia of undetermined etiology (HB in TABLE 2) was the diagnosis applied under these circumstances.

Results

As described in a preliminary report,[13] there was a marked difference in graft behavior between the 2 groups of infants. Rapid graft rejection was the fate of all grafts in infants receiving stored blood, but prolonged graft survival was noted in all the infants receiving fresh blood and skin from the same donor.

Four of the 6 infants in the stored-blood group rejected their grafts acutely, with necrosis and sloughing of both the epidermal and dermal elements in periods of less than 21 days (TABLE 1 and FIGURE 1). The 2 other infants, each receiving a graft from its mother, displayed a subacute modality of graft rejection, with persistence of the dermal, but not the epidermal, portions of the graft until at least the fourth postoperative week. In one of these patients (K.B.) epithelial necrosis was rapid and complete on postoperative day 13, but in the other case (M.C.) a more irregular, piecemeal necrosis of the epithelium took place between postoperative days 25 and 30. In both these cases re-epithelization by host epithelium took place superficial to the persisting dermal pad.

In the infants receiving exchanges of fresh blood and grafts of the corresponding blood donor's skin there was no ulceration or macroscopic evidence of graft necrosis at any stage (FIGURE 2). This made it difficult to define the exact survival time for grafts in this group. Edema, which was a characteristic feature of these grafts between the third and fourth postoperative weeks, subsided slowly.

Table 2

Composite Clinical and Investigative Data Relating to First-Set Homograft Survival in 12 Newborn Infants Following Exchange Transfusion*

Patients	Diagnosis	Blood groups		Exchange transfusions			Skin donor	Time of grafting (D.O.L.)	Graft survival (days)	Remarks
		Mother	Infant	Serial No.	Time (D.O.L.)	Vol. (ml.)				
Stored-blood recipients										
R.S. (Female)	EBF	B−	O+	1 2	3 4	460 510	Blood donor No. 1	4	17–20	Acute homograft rejection
I.N. (Female)	EBF	A−	O+	1 2 3 4	1 4 4 5	540 280 540 380	Blood donor No. 1	3	15–17	Acute homograft rejection
R.G. (Male)	EBF	A−	A+	1	1	380	Father	4	17–19	Acute homograft rejection
I.W. (Male)	Prem.	O−	O−	1 2	2 3	230 210	Father	2	12	Acute homograft rejection
K.B. (Male)	HB	O−	O+	1	2	400	Mother	3	13	Acute epithelial necrosis at P.O.D. 13; persistence of "dermal pad" until at least fourth postoperative week
M.C. (Male)	HB	O−	B+	1 2 3	3 4 4	140 200 410	Mother	4	25–30	Subacute rejection; patchy epithelial necrosis between P.O.D. 25 and 30 with more prolonged persistence of "dermal pad"

Fresh-blood recipients										
S.T. (Male)	HB	O+	B+	1 2 3	3 4 6	420 360 460	Blood donor No. 1	3	154	Excised *in toto* on P.O.D. 154. Cellular infiltration and FB giant-cell reaction suggest slow modified graft rejection
J.L. (Male)	Prem.	O+	A+	1	5	320	Blood donor	7	148+	Partly excised on P.O.D. 88. No definite histological evidence of graft rejection
M.D. (Male)	EBF	A−	O+	1 2	1 2	400 430	Blood donor	3	68	Same blood donor for both exchanges. Graft excised on P.O.D. 68, with no definite biopsy evidence of graft rejection
T.F. (Male)	Prem.	O−	B−	1	4	330	Blood donor	4	81	Excised at autopsy. Histological evidence of persistence of the graft epidermis, glands, and follicles, as well as dermis. Considerable cellular infiltration suggests modified graft rejection in progress
W.G. (Female)	EBF	O+	A+	1	2	410	Blood donor	2	125	Biopsy on P.O.D. 125 showed persistence of graft dermis, but replacement of epithelium by cells of female type
M.McG. (Male)	HB	A+	A+	1	3	500	Blood donor	3	76+	Macroscopically appeared intact, but undergoing contracture. No biopsy

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* Abbreviations: EBF, erythroblastosis fetalis; HB, hyperbilirubinemia of undetermined etiology; Prem., prematurity; D.O.L., day of life; P.O.D., postoperative day.

The subsequent course and behavior of the grafts in the fresh blood group have now been followed for a sufficient period of time to write a final evaluation of their fate. In attempting to evaluate the status of the 6 grafts that exhibited prolonged survival, the original macroscopic observations have now been supplemented with evidence from several graft biopsies and from second-set skin grafts in 3 of these children (S.T., J.L., and M.D.).

As far as macroscopic observation has gone, gradual contracture of 3 of the first-set grafts (J.L., W.G., and M.McG.) resulted ultimately, after periods of approximately 70 to 180 days, in appearances indistinguishable from that of scar tissue. Concerning the final appearance of the other 3 first-set grafts, no statement can be made, as they were excised *in toto* for histological study while still apparently surviving at postoperative days 68 (M.D.), 81 (T.F.), and 154 (S.T.).

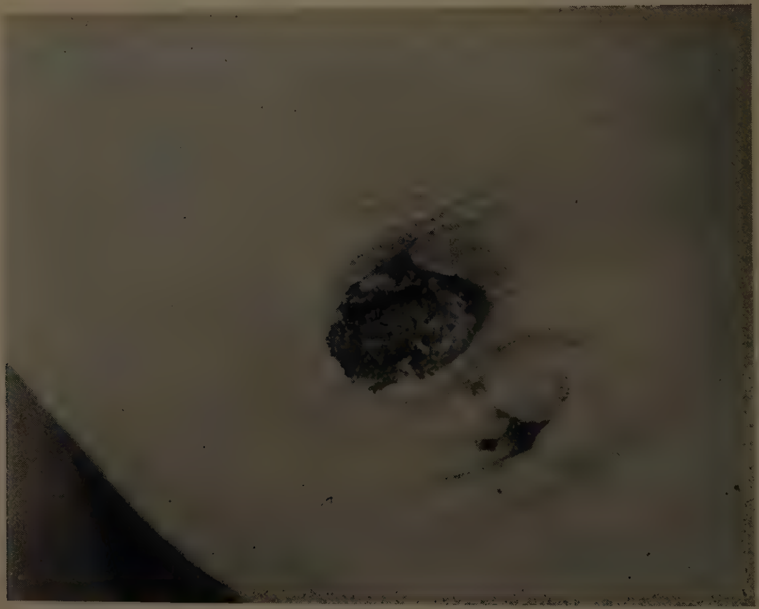

FIGURE 1. Patient I.W., stored-blood group. Day 13. Acute homograft rejection has occurred, with gangrene and sloughing of the graft. Umbilicus visible below graft.

Biopsy material included those 3 grafts removed *in toto* and partial biopsies at postoperative days 88 (J.L.) and 125 (W.G.) of 2 of 3 grafts (J.L., W.G., and M.McG.) allowed to remain indefinitely *in situ*. By contrasting this material with biopsies taken at varying times from the scars of 3 control infants (I.W., K.B., and M.G.*), who rejected their homografts, and with serial

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* In this context it should be noted how very few yardsticks of comparison are available. Since no extensive studies of homograft survival or of the second-set reaction in infancy have been attempted, we can use as a standard of reference only the first-set survival times in our own control group of infants, together with the similar periods of 21 days or less already reported by Peer[8] for acute homograft rejection in infants.

That the second-set reaction is not in abeyance in infancy is shown by our own observations of first-set and second-set rejection of mother's skin by a male infant (M.G.) with meningobiopsies from an autograft performed on one of us (C.D.W.), a satisfactory interpretation of the histological appearances was reached.

Second-set grafts from the original donors were used to close the skin defects resulting from the excision of the 2 first-set grafts removed on postoperative days 68 (M.D.) and 154 (S.T.). One other second-set graft was placed alongside a first-set graft (J.L.) when this graft was partially biopsied on postoperative day 88.

Both the histological evidence from the biopsies and the outcome of these 3 second-set skin grafts point to a substantial but incomplete and varying degree of tolerance in the 6 infants in this group.

Histologically, the evolution of the host reaction in these examples of prolonged graft survival was characterized in the later stages by scanty to moderate mononuclear cell infiltration (FIGURES 3, 4, and 5), foreign body giant-cell formation (FIGURE 6), and replacement fibrosis in the dermal remnants of the graft. However, the intensity and time of appearance of these changes varied from graft to graft (FIGURES 7, 8, and 9) indicating some difference in degrees of tolerance. One biopsy (W.G.) at postoperative day 125, from a piece of male skin grafted onto a female recipient, showed nuclear sex chromatin evidence of replacement of the graft epidermis by host epithelial cells at a stage when there was still only inconspicuous evidence (FIGURE 9) of cellular reaction in the persisting graft dermis. This modality of chronic graft rejection appears to offer a close analogy to the sequence of events in corneal transplants, in which it is known[15] that the host epithelium migrates to replace the graft epithelium, while the relatively well-tolerated corneal stroma persists for very long periods.

The results of the 3 second-set grafts are especially illuminating in regard to the spectrum of degrees of tolerance induced. The survival times of the grafts varied widely (TABLE 3), but 2 at least (J.L. and M.D.) were considerably in excess of what could reasonably be expected (*see* footnote to pages 408–409). The modalities of their rejection likewise differed: 1 graft (S.T.) suffered acute homograft rejection beginning on the tenth postoperative day; 1 (M.D.) displayed, between the fourth and fifth postoperative weeks, a subacute ulcerating type of epithelial rejection with persistence of the dermal remnant (FIGURE 10); and 1 (J.L.) mimicked in its behavior the even more prolonged nonulcerative type of rejection manifested by the first-set grafts in this group (FIGURE 11). Only a limited correlation was possible, in view of the small number of observations made, between the intensity of the first-set histological changes and the fate of the second-set grafts. The first-set graft (S.T.), removed as late as day 154, showed the most extensive cellular infiltration and giant-cell reaction of any of the biopsies (FIGURES 5 and 6), presumably indicative of an immune response close to or reaching its peak. The second-set graft performed at this time was the one that suffered the fastest rate of rejection, commencing on postoperative day 10. Neither of the first-set grafts from the infants with

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myelocele. Grafts performed on the fifth and fortieth days of life were rejected in 8 and 5 days, respectively. From these findings and from the data on first- and second-set human skin grafts already available in the literature,[15] it seems reasonable to expect that second-set survival times in infancy would probably not exceed 5 to 10 days unless some specific modifying factors were present.

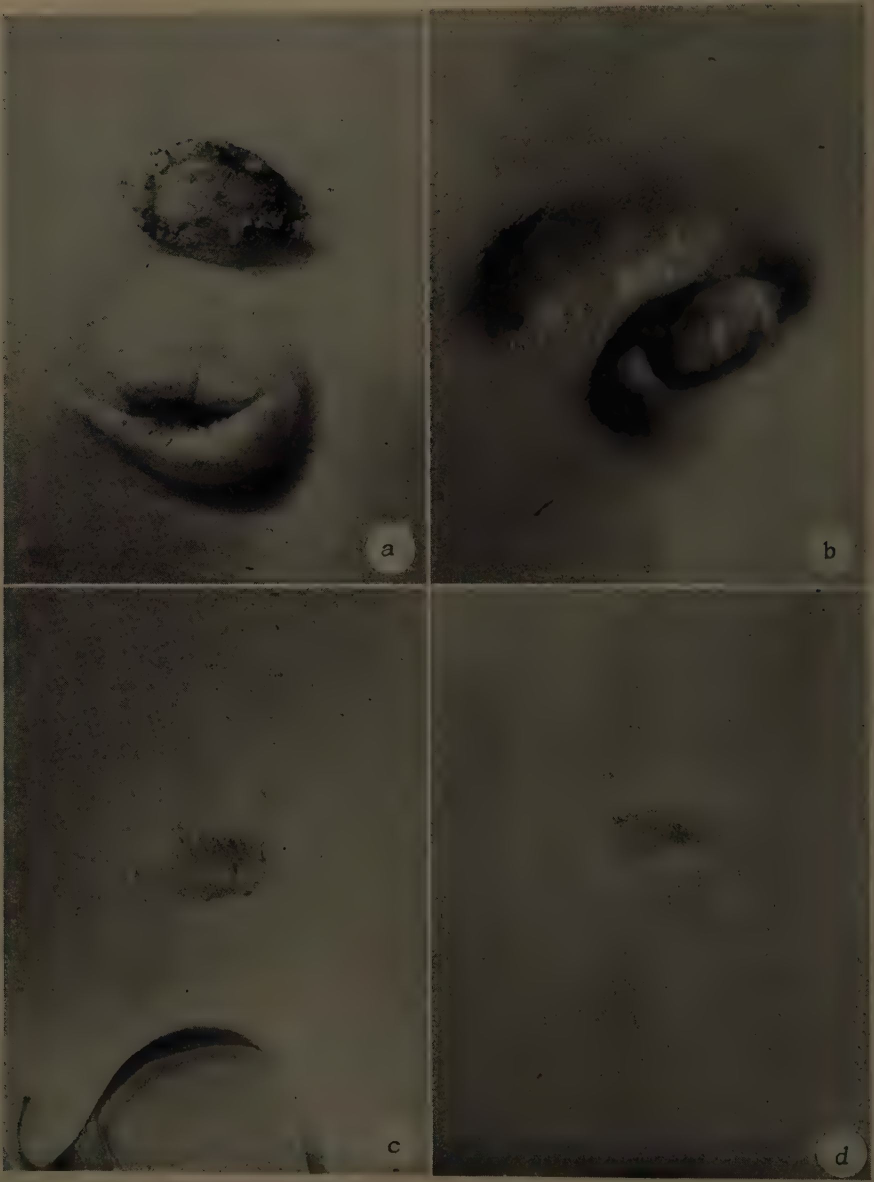

FIGURE 2. Patient T.F., fresh-blood group: (*a*) Day 11. Graft is healed in place. Note pigmented area to left of center. Umbilicus visible below graft. (*b*) Day 23. Edema of entire graft is present, without ulceration or necrosis. Umbilicus visible below graft. (*c*) Day 35. Edema is subsiding. Pigmented area persists. Thumb of examiner below graft. (*d*) Day 70. Edema is gone. Graft epithelium is almost indistinguishable from surrounding skin. Pigmented area persists. Umbilicus at lower left.

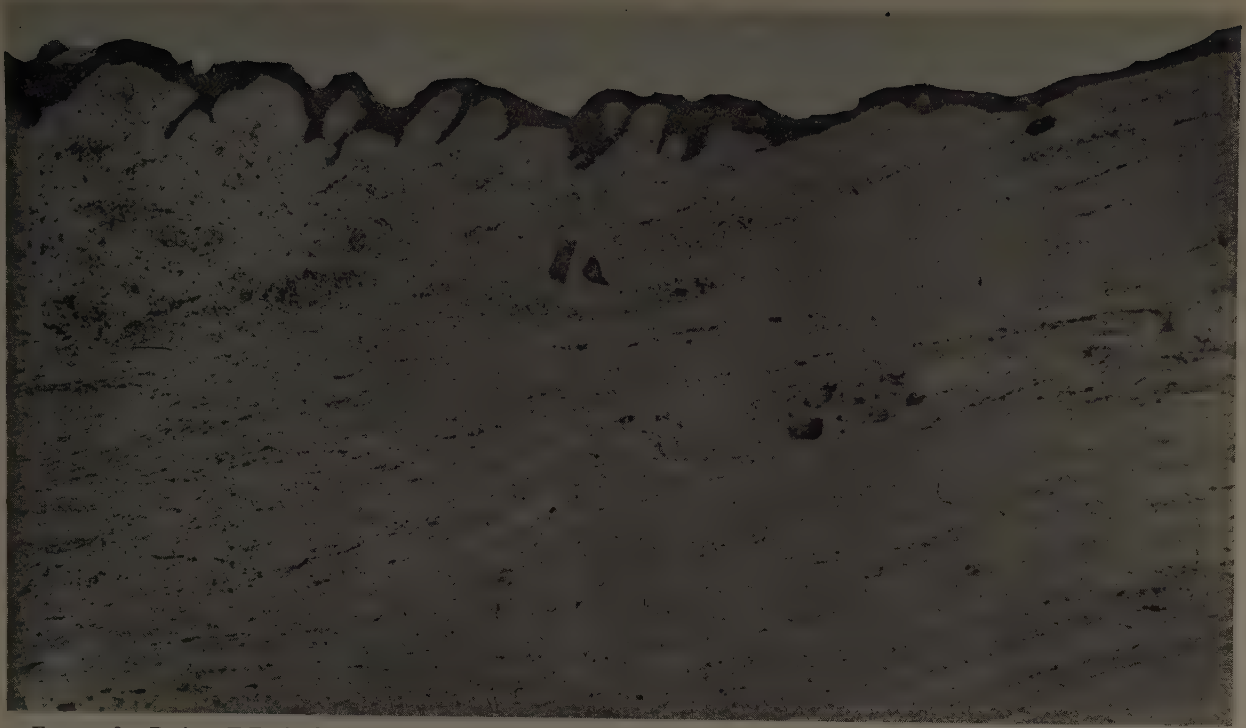

FIGURE 3. Patient T.F., fresh-blood group. Graft-host junction on day 81. The persistent graft (*left*) can be distinguished from the host (*right*) by the well-defined rete pegs of the graft epithelium. Moderate mononuclear cell infiltration is present in the graft dermis. ×35.

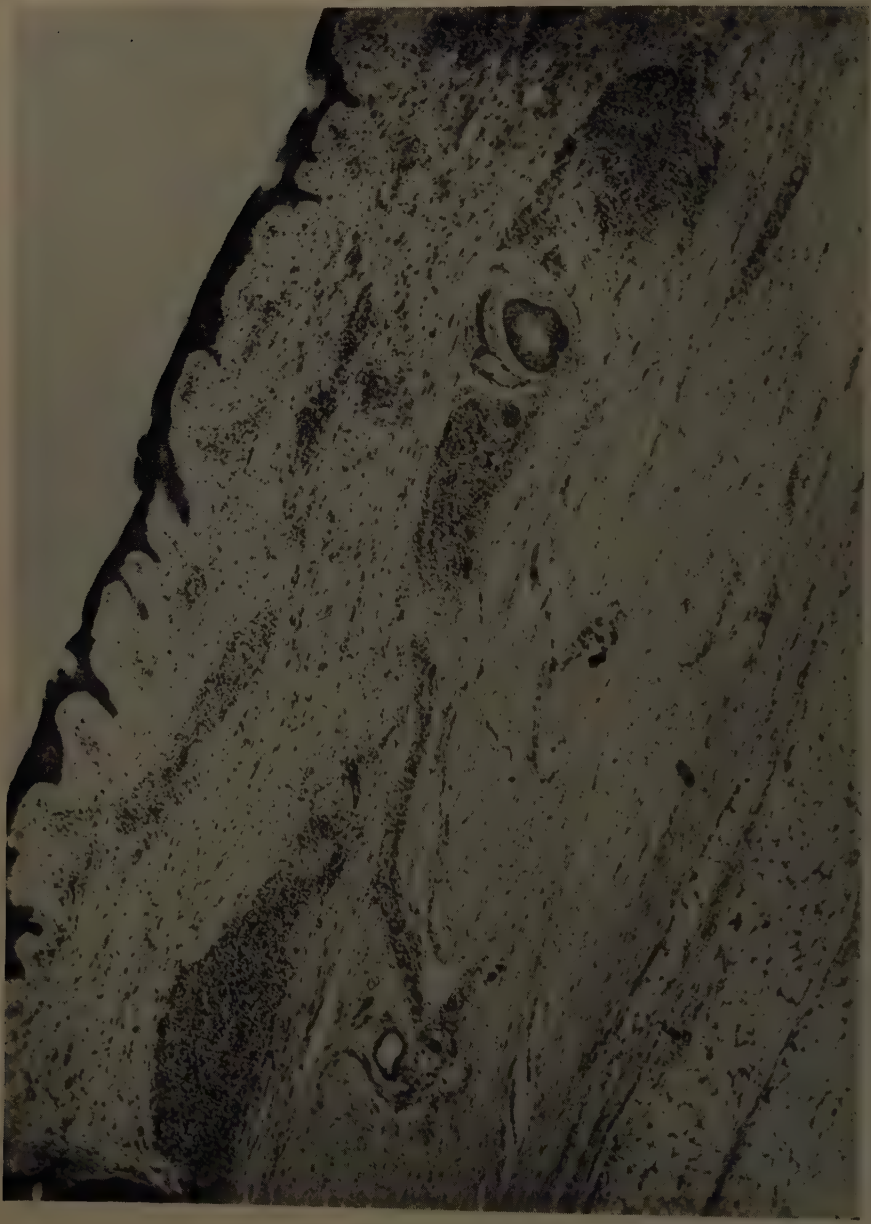

FIGURE 4. Patient T.F., fresh-blood group. Higher-power view of graft on day 81. A moderate mononuclear cell infiltration is present in the dermis. A hair follicle (*right of center*) persists in the graft dermis. ×100.

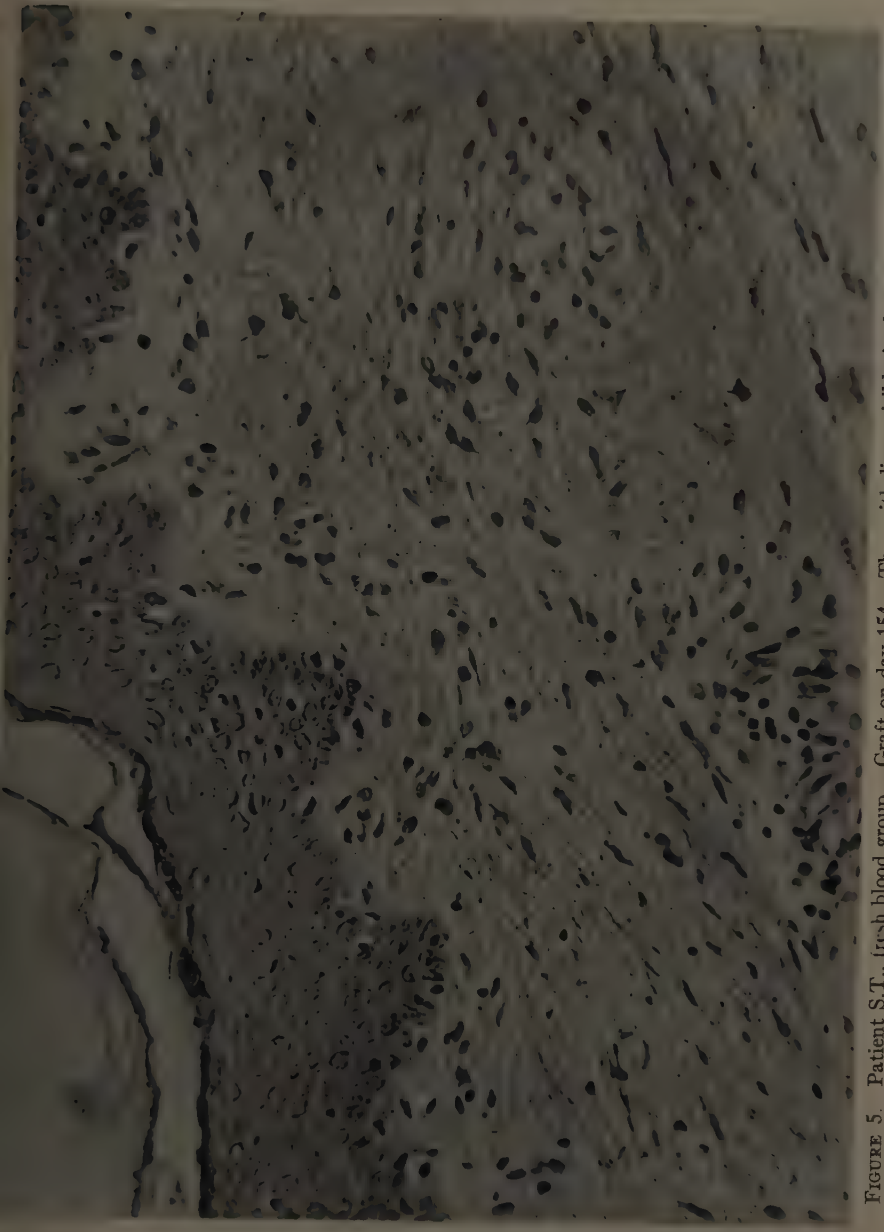

Figure 5. Patient S.T., fresh blood group. Graft on day 154. The epithelium visible is that of the graft. A relatively scanty mononuclear cell infiltrate is present in the dermis. ×100.

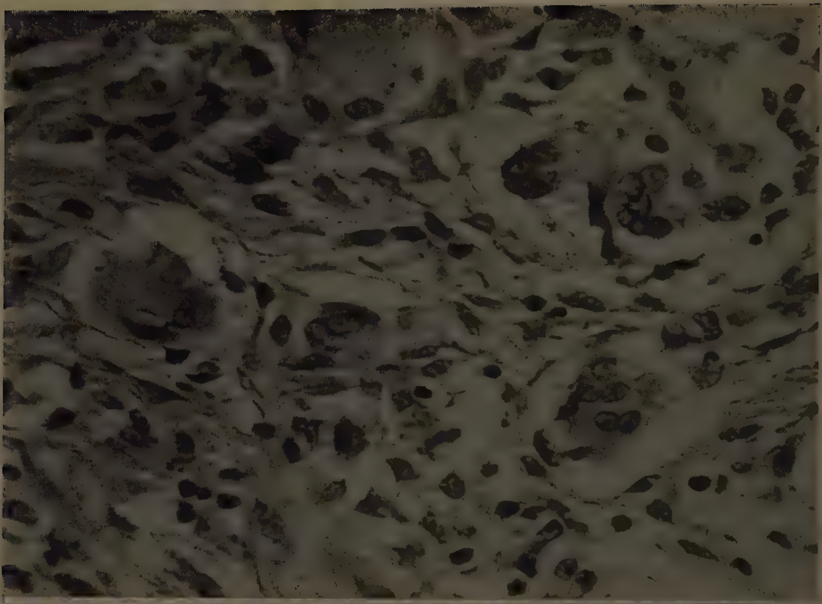

FIGURE 6. Patient S.T., fresh-blood group. Graft dermis, day 154. High-power view of foreign body giant-cell formation. ×430.

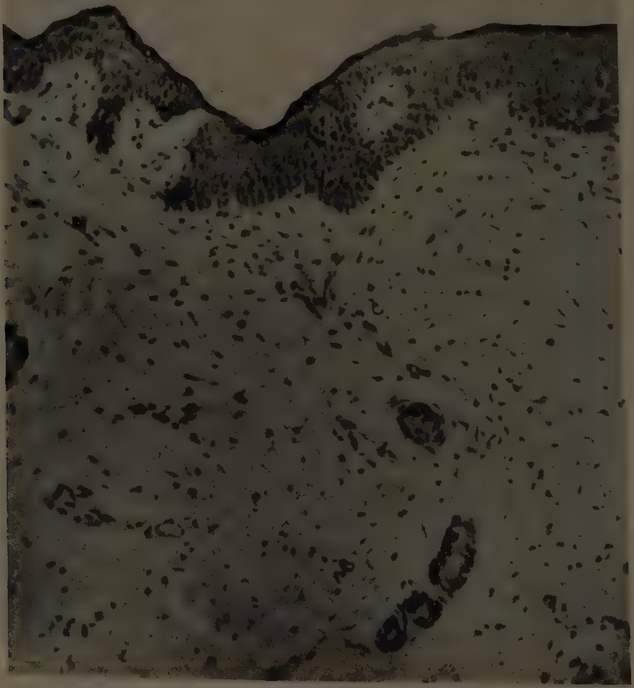

FIGURE 7. Patient M.D., fresh-blood group. Graft-host junction, day 68. The graft (*left*) can again be demarcated from the host (*right*) by the well-defined rete pegs of the graft epithelium. No mononuclear cell infiltration is present. ×100.

longer second-set survival times (M.D. and J.L.) had developed significant histological changes indicative of graft rejection by days 68 (FIGURE 7) or 88 (FIGURE 8), respectively.

In summary, the bulk of our evidence points to the fact that in no case did we succeed in inducing a complete degree of tolerance, although obviously a considerable blunting of the homograft reaction was achieved. In 3 of 6 cases (S.T., T.F., and W.G.) histological evidence was obtained suggesting that a slow modified form of rejection was in progress in these grafts. In 2 of the 6 first-set grafts (J.L. and M.McG.) and in one of the second-set grafts (J.L.) this interpretation was made on macroscopic evidence alone. In regard to the one other first-set graft (M.D.), excised completely on day 68 without histological signs of rejection, it cannot be said that it would or would not have been rejected if allowed to remain *in situ*. However, since the second-set

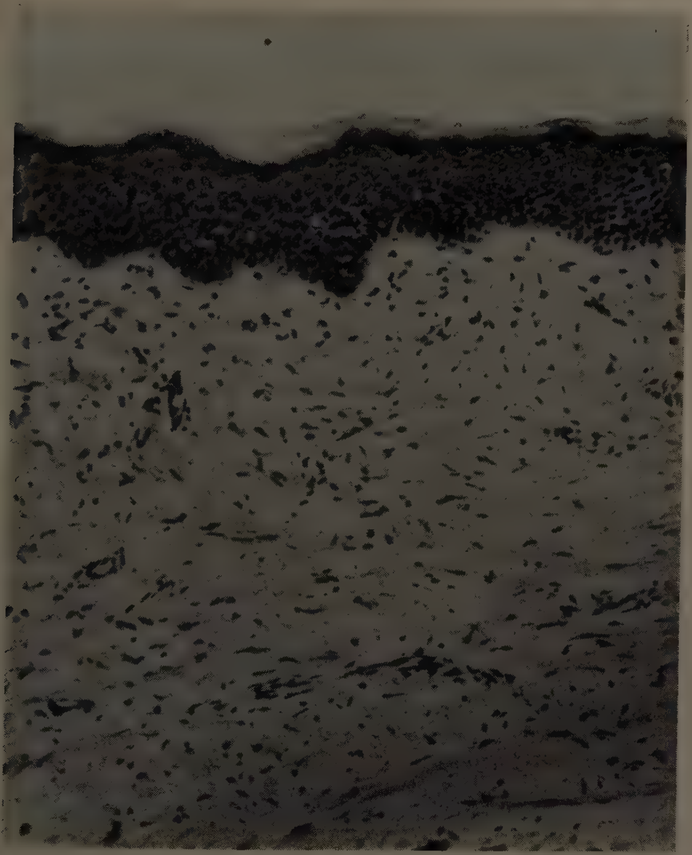

FIGURE 8. Patient J.L., fresh-blood group. Graft-host junction, day 88. Graft epithelium is on the left, host epithelium on the right. Graft epithelium characterized by rete pegs. No mononuclear cell infiltration is present in the dermis. ×100.

graft in this case was rejected after 4 weeks, it seems probable that the first-set graft would also have been rejected after a sufficient period of time.

No attempt was made in this study to explore the individual specificity of the homograft tolerance observed.

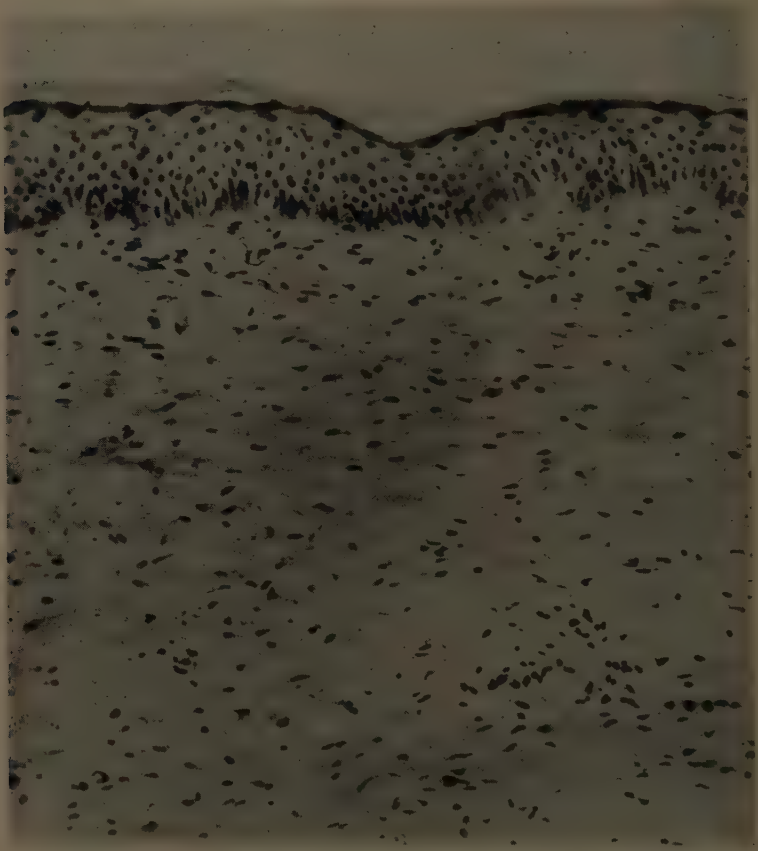

FIGURE 9. Patient W.G., fresh-blood group. Graft, day 125. Little or no mononuclear cell infiltration is present in the dermis. Nuclear chromatin studies showed replacement of chromatin positive graft epithelial cells with chromatin negative host epithelial cells. ×100.

The significance of the evidence, presented by the control group, that the human infant already possesses at birth a mature homograft rejection mechanism, will be emphasized subsequently.

One other point of interest was that, in spite of large-scale transfer of leukocytes to the six infants displaying prolonged graft survival, passive transfer of delayed cutaneous hypersensitivity to histoplasmin or tuberculin was not noted. Five of the six blood donors were sensitive to histoplasmin and four to tuberculin (PPD). Six of the 6 infants tested for histoplasmosis and 4 of the 4 tested for tuberculin had negative skin reactions. The tests were done between the ninth and twenty-eighth day after transfusion.

In none of the children displaying prolonged graft survival was there any immediate or early deleterious reaction that might have been attributable either to the grafting procedure or to the leukocyte transfer, and nothing comparable to the phenomenon of runt disease as elicited in certain animal ex-

TABLE 3
COMPARISON OF FATE OF FIRST- AND SECOND-SET SKIN HOMOGRAFTS IN 3 NEONATAL LEUKOCYTE RECIPIENTS

Patient	Time of grafting (day of life)	Survival (days)	Remarks
S.T.	3	154 (Excised)	Extensive infiltration of graft by mononuclear cells. Suggests slow modified homograft rejection in progress
	157	10 (Rejected)	Acute homograft rejection
M.D.	3	68 (Excised)	No histological evidence of rejection
	71	28–34 (Rejected)	Slow rejection
J.L.	7	118+	Partly excised at 88 days. No histological evidence of rejection
	95	30+	Both first-set and second-set grafts appear to be surviving

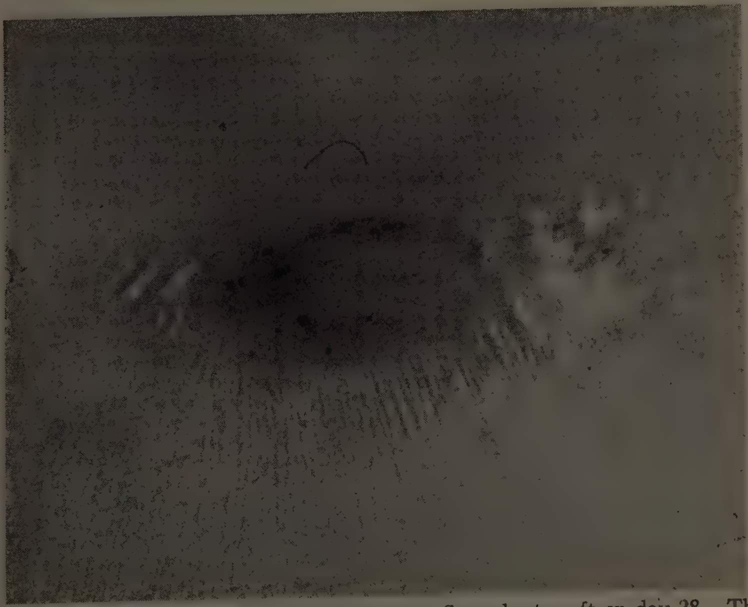

FIGURE 10. Patient M.D., fresh-blood group. Second-set graft on day 28. The graft epithelium is undergoing a subacute ulcerative rejection. The dermal remnants persist.

periments was observed. However, late major infective episodes occurred in 2 of the children in the fresh-blood group. One of these children (M.D.) developed bronchopneumonia at the age of 6 weeks and *Hemophilus influenzae* meningitis at the age of 10 months. He recovered rapidly and uneventfully from both these infections with conventional forms of therapy. The other child (T.F.) who was thriving and robust up to the age of 3 months died with the "unexpected sudden-death syndrome" of infancy[16] and with the postmortem findings so commonly present in this syndrome of tracheitis and interstitial pneumonitis (FIGURE 12). Normally developed and active germinal follicles were present both in his lymph nodes (FIGURE 13) and spleen (FIGURE 14), in contrast to the lymphoid atrophy that is typical of the runting syndrome. The liver was normal. There was no evidence to show whether these serious infective complications in 2 children were merely coincidental or more closely related to the neonatal transfer of leukocytes by exchange transfusion employing currently accepted practices. This is an important question requiring further discussion.

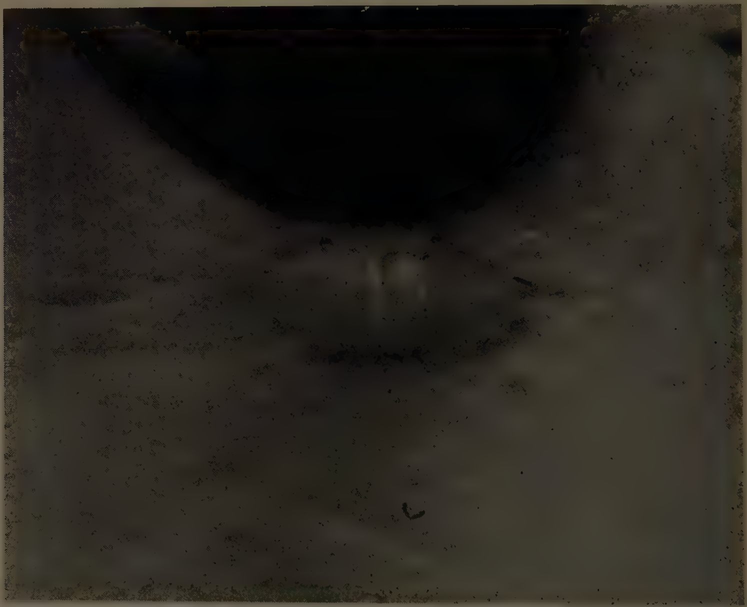

FIGURE 11. Patient J.L., fresh-blood group. Second-set graft, day 28. This graft show no ulceration, but edema, similar to that seen in the first-set graft at 4 weeks, is present.

Discussion and Conclusions

We conclude from our observations on these homografts that some form of decreased reactivity has been evoked by the fresh-blood exchanges described and, further, that this altered reactivity is incomplete in nature. Although there is no direct evidence for the assumption, it seems probable that the leukocyte fraction in the fresh-blood transfusions was responsible for the effect obtained.

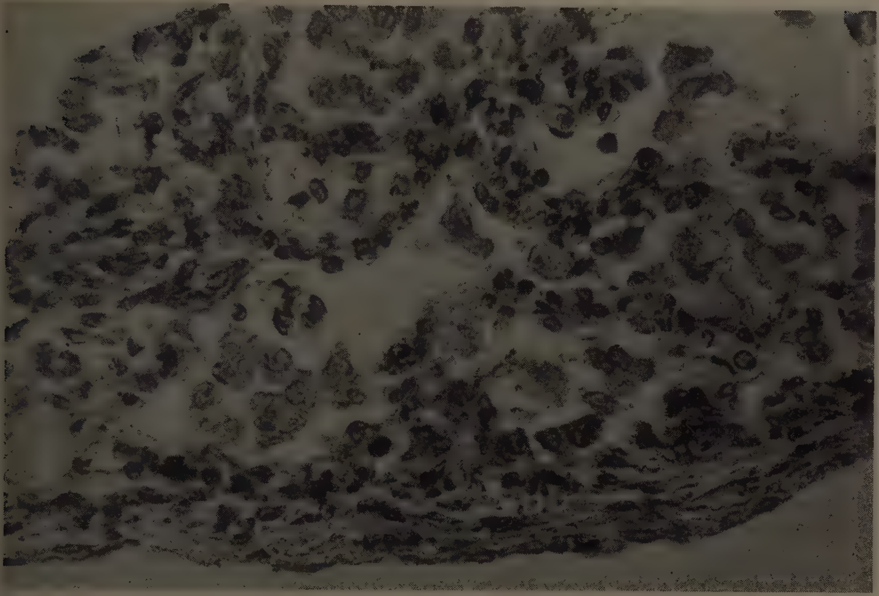

FIGURE 12. Patient T.F., fresh-blood group. Lungs, post mortem. A mononuclear infiltrate is present in the alveoli and interstitial tissue. ×393.5.

FIGURE 13. Patient T.F., fresh-blood group. Spleen, post mortem. Normal differentiation into red and white pulp is apparent. ×35.

It has been suggested[17] that multiple exchanges with blood of different individuals of a particular blood group might induce a state of homograft tolerance toward skin from any other donor belonging to the same blood group. However, the necessity for multiple-exchange transfusions in several of our children did not appear to affect the fate of the graft. Multiple exchanges with stored blood from different donors prevented neither acute graft rejection in 4 infants (R.S., I.N., I.W., and M.C.) in the control group nor prolonged graft survival, following an initial transfusion with the skin donor's fresh blood, in the only infant (S.T.) in the second group who required additional exchanges.

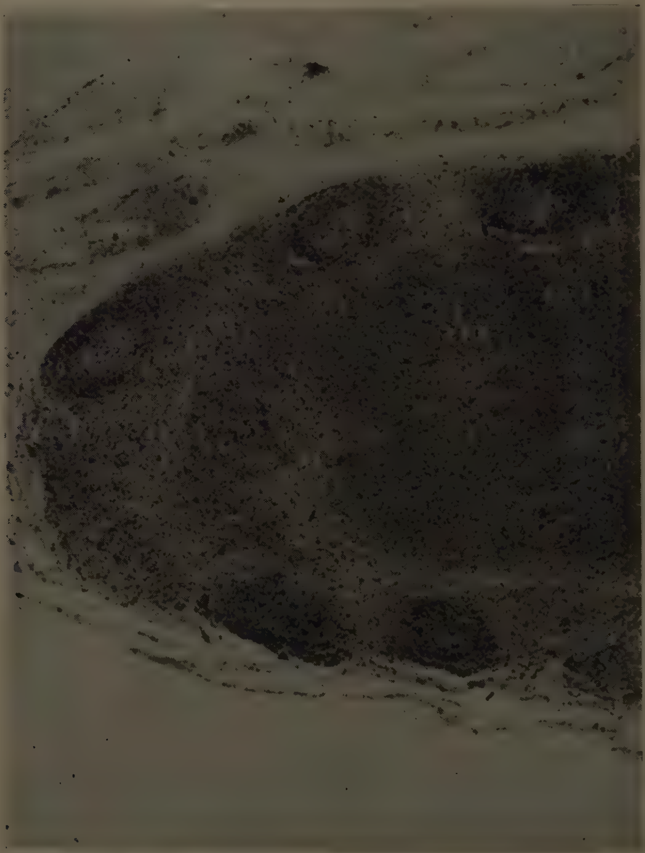

FIGURE 14. Patient T.F., fresh-blood group. Lymph node, post mortem. The germinal follicles are normal. There is no evidence of lymphoid atrophy. ×35.

Within the short range of variation covered by the timing of transfusions or skin grafting in relation to the time of birth, there was no suggestion in the results obtained that the precise day of interference had any effect on the ultimate fate of the grafts. The underlying etiology of the hyperbilirubinemia likewise appeared to be irrelevant to the effects of transfusion on graft survival.

Although male-to-female grafts are not tolerated in inbred strains of mice,[8] a sex difference between donor and host did not prevent prolonged homograft survival in the one instance (W.G.) of male-to-female grafting in this group.

Evidence of infant tolerance toward maternal grafts was meager (K.B. and M.C.), but the slight modification in the course of rejection of these 2 maternal grafts may be analogous to the tolerance to maternal grafts reported in some infants by Peer.[8]

Perhaps the most surprising feature of the altered reactivity to homografts found in this study is not that it should be incomplete, but that it could be elicited at all, in view of the comparative developmental maturity of the human infant at birth. This maturity is pointed up even further by the fact that 1 premature infant (I.W.) in the control group, weighing only 3 lb. at birth; at an estimated gestational age of 32 weeks, was fully capable of rejecting a graft of his father's skin in as short a period as 12 days. Our results, therefore, are difficult to interpret in the face of current concepts of a critical period of immunological maturation since, if a premature infant already possesses a mature homograft rejection response, the full-term infant should have already progressed well beyond any critical period for the induction of tolerance.

Obviously the altered reactivity described in this study is not strictly analogous to the homograft tolerance or related runting condition that can be elicited in certain species by leukocyte transfer prior to the critical period. It is equally apparent, from the findings in the lymph nodes and spleen of the patient who died with pneumonia and an intact graft, that the tolerance effected by fresh blood transfusions is not mediated by production of lymphoid hypoplasia in the host. Atrophy or hypoplasia of lymphoid tissues is one of the characteristic features of the tolerance associated with runt disease in animals,[2] and has been thought to be due to transplanted cells with mature immunological capacities colonizing and "rejecting" the lymphoid tissues of the host.

In this context it may be relevant to note that hitherto in animal experiments cells of lymphopoietic or hematopoietic lineage have been the most effective in eliciting homograft tolerance, although other types of cells, both normal and neoplastic, may do so.[1] It may well turn out that the dosage, route of administration,[19] and type of cells utilized are of more importance in the induction of this phenomenon than any arbitrary critical period of immunological maturity. Alternatively, in individuals beyond this critical period there still may be unrecognized inimical effects of leukocyte transfer upon mature immunological mechanisms. This possibility may have some relevance to the late infective conditions encountered in 2 patients (M.D. and T.F.) in this study. However, before dismissing the possibility that 2 of our patients could have had these serious infections by chance, it must be remembered that fresh-blood exchange transfusions have been widely performed in recent years without any recognized untoward sequelae. Before categorically condemning this practice on the basis of the few observations of the present investigation, which was strictly limited in scope, a far more detailed study will be required of the immunological capacity and susceptibility to infection of infants transfused with either fresh blood or stored blood.

In any future investigation of immunological deficiency in infants with the clinical picture of erythroblastosis the possible influence of other variables in this clinical situation must be considered. These include the depression of bone marrow function that has been observed occasionally to last for several months following exchange transfusion,[20,21] the diversion of fetal reticuloendothelial tissues to extramedullary erythropoiesis, and humoral or cellular influences from the mother on fetal reticuloendothelial functions other than those of erythropoiesis. In erythroblastotic infants in the control group acute graft rejection was not suppressed, however, even by multiple exchange transfusions, so that we have discounted all of these possible influences, together with any effects of hyperbilirubinemia or the exchange procedure itself, insofar as inhibiting the homograft reaction is concerned. It still may be somewhat premature, however, to discount them in regard to later susceptibility to infection in infancy.

What the results of this study do indicate is that leukocyte transfer in the human neonate can interfere in some unknown manner with at least 1 normal immunological mechanism: that is, the one concerned in homograft rejection. Whether this interference could lead, in addition, to an increased susceptibility to infectious disease in infancy is a question that warrants future investigation and is engaging our further attention.

In regard to the observations on delayed cutaneous hypersensitivity it is well known that this phenomenon can be passively conferred by leukocyte transfer either by the intradermal[22] or intravenous[23] route, and that passively conferred hypersensitivity in man is more often of several months' duration,[24] rather than one of weeks or days, as is the case in guinea pigs. The failure in this study of transfused leukocytes to confer delayed cutaneous hypersensitivity to these infants by the intravenous route may be related closely to the similar failure in the neonatal period of passive transfer of delayed cutaneous hypersensitivity by intradermal leukocyte injections (R. Good, personal communication). If infants in this age group provide an unsuitable milieu for eliciting delayed hypersensitivity responses, it certainly does not relate to immaturity of their homograft rejection mechanisms. In spite, therefore, of the many analogies[25] that can be found between delayed hypersensitivity and the homograft reaction, the behavior of the newborn human infant tends to dissociate the mechanisms of these 2 phenomena.

Summary

Twelve newborn human infants were test-homografted with adult skin following exchange transfusion for neonatal jaundice. Rapid graft rejection was observed in 6 infants who were exchanged with blood stored for 48 hours or more. By contrast, marked prolongation of graft survival was found in 6 infants who received transfusions of fresh blood and skin from the corresponding blood donor. Evidence was obtained that this form of homograft tolerance is incomplete. The mechanism of its production is unknown, but it appears to differ from the tolerance induced by leukocyte transfer to animals in an "adaptive phase" of immunological maturation. The newborn human infant already displays a highly developed homograft rejection mechanism.

Passive transfer of delayed cutaneous hypersensitivity, on the other hand, was not elicited at this age following massive intravenous leukocyte transfer from histoplasmin or tuberculin-positive donors.

References

1. Billingham, R. E., L. Brent & P. B. Medawar. 1956. Quantitative studies on tissue transplantation immunity. III. Actively acquired tolerance. Phil. Trans. Roy. Soc. London. Ser. **B239:** 357.
2. Billingham, R. E. 1958. Studies on the reaction of injected homologous lymphoid tissue cells against the host. Ann. N. Y. Acad. Sci. **73**(3): 782.
3. Billingham, R. E., L. Brent & P. B. Medawar. 1955. Acquired tolerance of skin homografts. Ann. N. Y. Acad. Sci. **59**(3): 409.
4. Woodruff, M. F. A. 1957. Postpartum induction of tolerance to homologous skin in rats. Ann. N. Y. Acad. Sci. **64**(5): 792.
5. Billingham, R. E. & L. Brent. 1957. Acquired tolerance in newborn mice. Ann. N. Y. Acad. Sci. **69**(4): 678.
6. Woodruff, M. F. A. & L. O. Simpson. 1955. Induction of tolerance to skin homografts in rats by injection of cells from prospective donor soon after birth. Brit. J. Exptl. Pathol. **36:** 494.
7. Woodruff, M. F. A. 1957. Can tolerance to homologous skin be induced in the human infant at birth? Transplantation Bull. **4:** 26.
8. Peer, L. A. 1957. Behavior of skin grafts interchanged between parents and infants. Transplantation Bull. **4:** 109.
9. Good, R. A. 1958. Discussion of R. E. Billingham: Runt disease as a complication of inducing tolerance to homologous tissues with lymphoid cells. *In* Immunity and Resistance to Infection in Early Infancy. : 59. 29th Ross Pediatric Research Conference. Ross Laboratories. Columbus, Ohio.
10. Allen, T. H., Jr. & L. K. Diamond. 1957. Erythroblastosis Fetalis, Including Exchange Transfusion Technique. : 64. Little, Brown. Boston, Mass.
11. Wheeler, W. E. & J. P. Ambuel. 1957. The efficient use of exchange transfusions in the treatment of erythroblastosis. Pediat. Clin. N. Am. : 383.
12. Vaughan, V. C. 1959. Management of hemolytic disease of the newborn infant. J. Pediat. **54:** 586.
13. Fowler, R., Jr., W. K. Schubert & C. D. West. 1959. Survival of skin homografts following exchange transfusion for neonatal jaundice. A.M.A. J. Diseases Children. **98:** 585.
14. Rogers, B. O. 1957. The genetics of skin homotransplantation in the human. Ann. N. Y. Acad. Sci. **64**(5): 741.
15. Maumenee, A. E. 1955. The immune concept: its relation to corneal homotransplantation. Ann. N. Y. Acad. Sci. **59**(3): 453.
16. Adelson, L. & E. R. Kinney. 1956. Sudden and unexpected death in infancy and in childhood. Pediatrics. **17:** 663.
17. Albert, F. & G. Lejeune-Ledant, P. Moureau & A. André. 1959. Does a total transfusion done shortly after birth induce a state of tolerance in receptor? F. Albert and P. B. Medawar, Eds. *In* Biological Problems of Grafting. : 369. Thomas. Springfield, Ill.
18. Eichwald, E. J., C. R. Silmser & N. Wheeler. 1957. The genetics of skin grafting. Ann. N. Y. Acad. Sci. **64**(5): 737.
19. Billingham, R. E. & L. Brent. 1956. Acquired tolerance of foreign cells in newborn animals. Proc. Roy. Soc. London. **B146:** 78.
20. Hyman, C. B. & P. Sturgeon. 1955. Observations on convalescent phase of erythroblastosis fetalis. Pediatrics. **16:** 15.
21. Giblett, E. R., J. E. Varela & C. A. Finch. 1956. Damage of bone marrow due to RH antibody. Pediatrics. **17:** 37.
22. Chase, M. W. 1945. Cellular transfer of cutaneous hypersensitivity to tuberculin. Proc. Soc. Exptl. Biol. Med. **59:** 134.
23. Metaxas, M. N. & M. Metaxas-Buehler. 1955. Studies on cellular transfer of tuberculin sensitivity in the guinea pig. J. Immunol. **75:** 333.
24. Lawrence, H. S. 1959. Delayed hypersensitivity and the behavior of the cellular transfer system in animal and man. *In* Mechanisms of Hypersensitivity. : 453. Henry Ford Hospital Intern. Symposium.
25. Lawrence, H. S. 1959. Homograft sensitivity. Physiol. Rev. **39:** 811.

Discussion of the Paper

ALBERT: We have been interested for two years now in the possibility of inducing tolerance in children who have total or nearly total transfusion at birth.

Lejeune-Ledant, who is my collaborator, studied four of these cases very accurately. He started first with experimental work, proceeding in the same manner as Puza and Gombos, who used total exchange transfusions in young dogs. He obtained the same results with the induction of tolerance as had Puza and Gombos.

Therefore, we turned our attention to children and observed four different cases. We now have 4 children who had transfusions. We placed them in 2 different groups. The first case, which we presented at the Liége Conference in 1959, concerned a boy of 6 years who had congenital erythroblastosis and had transfusions from several blood donors at birth or soon thereafter. When a child is transfused for erythroblastosis at the Transfusion Department at Liége, 2 different donors are used. One of the donors, a female, died between the date of the birth of the boy and the child's sixth year, so we had only the choice of the second donor, but the boy demonstrated a very important, if not a total, tolerance to the skin homograft taken from this donor and used as a test of the child's tolerance. When we published a description of the case at the Liége Conference, we asked the Transfusion Department to allow us to review again all the details of the problem. Finally, it was demonstrated that at birth the boy had received only the blood of the female donor, who died, and never had received the blood of the man who was the donor of the skin homograft!

I found this rather amusing, but when we started to investigate this case further, we found that as the boy had had a very severe anemia, he had had 2 other transfusions given him during 2 or 3 weeks after his birth from 2 other donors, so that he had 3 consistent transfusions from 3 different donors, all of the same blood group, of course.

The results in this case may be important. The test must be repeated, of course, but it is not so easy to find such cases. It might be possible that a child who was polytransfused at birth with plural blood from different donors might become tolerant to homografts from another donor of the same blood group; if that could be demonstrated more conclusively, it would be extremely important indeed in future clinical applicability to a number of transplantation problems.

We had 3 other cases of total transfusion at birth. These 3 children, 1 boy and 2 girls, I think, were recently tested with skin homografts from 1 of their donors; one child was tested at the age of 3 years and the other 2 children at 6 years of age. As a result, we now have 3 different results: (1) a nearly total tolerance after 282 days of postoperative observation in a girl of 6 years; (2) a very important but not really total tolerance, I think, in another girl of 6 years; and, by way of contrast, (3) a normal breakdown after 10 or 14 days in the boy of 3 years.

All studies were done to see if there was any agammaglobulinemia or any uremia in order to be certain that there was no other explanation possible for the partial or total tolerance that was observed.

I think that these human experiments, if you can call them experiments, must be continued because it would be very interesting to know whether children who have been transfused at birth, or very near to the moment of birth, could become tolerant of subsequently transplanted homografts taken from the same original donors or, eventually, if plural blood is used from many donors of the same blood group, whether the subjects could become tolerant of any tissue obtained from members of the same blood group who were not, however, original donors in the plural transfusions given at birth. I do not know if children at birth have a certain immunological immaturity, because it is generally difficult to investigate children at this age. If we have true tolerance, however, one can see that the tolerance persists for a very long period of time: 3 children who were subsequently challenged with a skin homograft in our study were 6 years of age.

KOPROWSKI: When you mentioned that transfusions were given at birth, did you mean within hours after birth?

ALBERT: Yes.

KOPROWSKI: In all four cases?

ALBERT: In all four cases, yes.

KOPROWSKI: As for the boy who received the three transfusions, were the second and third transfusions given shortly thereafter?

ALBERT: Within the first three weeks.

SILVERS: In light of what we know about graft versus host reactions, are you not concerned about injecting leukocyte concentrates into these children? Is it not possible that a graft against host reaction will result? I believe that Woodruff abandoned his projects along this line when he realized that this might be of some danger. How has Fowler felt about this?

ROBERT A. GOOD: We began similar studies about 5 years ago when Michael Woodruff began his investigations at that time, and the extent of our studies and the conclusions drawn from them are essentially the same as those just described, except that we tested our homografts when the babies were between 4 months and 1 year of age. The results of our studies were that we had 2 babies with a very prolonged survival of the homograft or a very delayed slough. I think that these grafts were ultimately replaced by a gradual creeping substitution of surrounding host tissues.

One of the other babies had what seemed to be a normal rejection in spite of the previous exchange transfusion, and one of the babies had a delayed but complete rejection with a resultant granulating host surface. We abandoned our studies largely for the same reason that Woodruff did when Brent and Billingham and others made known their observations on the hazards of the administration of intact adult cells to young animals. I think that it is extremely interesting and very important to pediatricians that two apparent untoward reactions may have resulted, not necessarily from the studies that were carried out, but from the exchange transfusions in which the blood was taken early and handled very carefully.

We have done some subsequent work in attempting to transfer delayed allergy to these babies, which, along with immunological studies, indicates to us that these neonatal infants, although they can reject skin homografts, are immunologically aberrant. Therefore I think the important thing to result from this discussion is that we who use exchange transfusion should be very careful perhaps to discard the white cells rather than to try to inject them.

CHASE: I have only one bit of information that might be borne in mind. Newborn babies whose skin is painted with poison ivy extract are found to be sensitive between the sixth and the tenth day. Therefore, at birth, the human infant is capable of undergoing allergization of the delayed type.

R. L. WALFORD: We have been interested from a somewhat different angle in the response of individuals to the injection of living white cells, and have taken advantage of the system of cardiac surgery presently in vogue where infants or children and young adults are injected during surgical correction of congenital cardiac defects with what are essentially massive transfusions, 18 to 20 units of fresh whole blood in which the leukocytes are, by and large, quite viable. We have noted in this condition that, at about the eighth to tenth day after the transfusion of such leukocytes, in children and young adults one can demonstrate in 25 to 50 per cent of the cases the presence of both complete and considerably incomplete antibodies reacting against white blood cells.

I have a further question. I was interested in the granulomatous reaction that Fowler showed in their studies. Hildemann and I observed a granulomatous reaction, as he mentioned, but this seemed to be associated with the foreign-body reaction against extruded hair material from the hamster. Of course, there is much more hair in the hamster than in the human, and I wonder whether, in your granulomas, you noted any relation to the hair shaft material.

CINADER: It seems to me that after exchange transfusion one could examine whether one has induced tolerance to haptoglobin. As you know, there are three genetic types of haptoglobin, and you are very likely to have in this a very adequate test system. I wonder whether Fowler has considered using this test system to study the induced tolerance in his experiments.

JOSÉ VIÑAS (Barcelona, Spain): I have two questions. First, were Fowler's children given exchange transfusions for therapeutic reasons? I mean, were they sturdy children or not?

Second, as to the mild anemia that sometimes is seen in children who have been exchange-transfused at birth for incompatibility: could it be an expression of the transfused adult, immunologically competent cells that have been surviving for some time there?

R. FOWLER, JR.: It was certainly very interesting to hear from Albert about that very long survival in the girl and also, to hear again, from Good, the details of the survivals in his cases.

Good's suggestion that we might now abandon giving white cells is something that I shall discuss in detail, together with Silver's question as to whether we considered the possible risks before we went into this.

We did consider them, and were perfectly aware of Woodruff's cautions. At the time those cautions were framed, the type of thinking surrounding this problem was that if one gives reticuloendothelial-type cells with immunological capacity prior to a certain critical period, one will induce tolerance and, as a result of that tolerance, one may produce "runt" disease or some other harmful effect. However, once the animal has passed beyond that critical period, one will not produce tolerance and, *ipso facto*, one will not produce "runt" disease.

Before we started this, we were at pains to make certain that there was considerable medical precedent for what we were doing.

In recent years there have been considerable trends and modifications in the practice of exchange transfusion, the most notable being the greater urge toward using fresh blood wherever possible. There are practical reasons of great advantage to the infant for using fresh blood, and these we still respect.

The other feature is the trend toward plastic and siliconized transfusion equipment, which almost certainly guarantees that larger numbers of viable leukocytes are being transfused now than in former years.

Before we rashly decide to abandon fresh blood exchanges on the basis of these two results of ours, we must point out that this trend of giving fresh blood has been very widely practiced in recent years without any apparent untoward effects and that, since encountering these two infective complications, we have been at great pains to inquire among our colleagues whether they have experienced this susceptibility to infection that we have suggested might be more than a coincidental phenomenon. Thus far we have met very little encouragement in thinking that this is really the case. However, because of its potential significance, I think that this question should be pursued, not by abandoning the use of fresh blood, but by really doing immunological studies of two groups of infants: those receiving fresh blood and those receiving stored blood, and by charting the incidence in the first twelve months of life of infectious disease in these two groups. That would be my attitude to that question.

I thank Chase for mentioning the interesting feature of actively acquired delayed hypersensitivity to poison ivy in the newborn, which was unknown to me.

Walford commented about the relations of the foreign body giant cells to hair shafts, but we were not convinced that the hair had anything to do with this reaction in our grafts. Our first indication of this foreign body giant cell reaction was our pathologist's report that this was associated with buried silk suture fibers, but we disagree with this interpretation because the foreign body reaction is diffuse throughout the dermis of some of the chronically rejected grafts, and we think it is a facet of this type of chronic graft rejection.

In regard to Cinader's suggestion about haptoglobin, I have not considered using that as a test system; I thank him for the suggestion.

In regard to Viñas's question as to whether we performed these exchange transfusions for therapeutic reasons, the answer is "Yes."

Viñas also raised a very interesting point about the late anemia sometimes observed following exchange transfusion. This, apparently, is a rare phenomenon, but it has produced two papers in the pediatric literature. The thought has been expressed that this is due to a chronic effect of maternal RH antibody, continuing a depression of bone marrow activity in the host; this, I think, is another factor that must be considered when we raise the question of investigation of these infants for possible susceptibility to infectious disease.

If there is any inimical effect on their immunological mechanisms, how much of it is due to the transfusion procedure? How much is due to leukocytes? How much is due to residual effects of their basic disease, erythroblastosis or the hyperbilirubinemia for which exchange transfusion was then performed? There may still be depressive effects of maternal antibody. There may be effects that we do not yet recognize, but that were suggested a number of years ago by Billingham: that in some erythroblasts, maternal cells may even cross the placental barrier and be responsible for some immunological damage in the fetus.

Upon purely anatomical or pathological grounds, one might consider whether diversion of fetal lymphopoietic tissue to erythropoietic functions might in some way reduce immunological capacity early in neonatal life.

This is a very complex situation under study, and I can only urge that it receive the attention it deserves.

THE EFFECTS OF NUCLEIC ACIDS ON HOMOGRAFT TOLERANCE*

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Introduction

Although Fomon[1] described the Egyptian barkers of 3500 B.C. as having a limited number of successful skin transplantation procedures, the Twentieth Century surgeon employing the most efficient methodology is still observing a consistent sloughing and demise of a high percentage of skin homotransplants. It should be stressed that we are not cognizant of the seemingly infinite mechanics of the homograft reaction. Which in the constellation of intricate antigens invokes this response, and which are the antibodies that contribute to it, also are facts unknown to modern researchers.

Many investigators[2-15] have written extensive reviews on homotransplantation. These reviews not only inform the reader of advances made in the field, but also indicate the many links still missing in our understanding of homotransplantation immunity.

The classic experiments performed by Medawar[16] clearly demonstrated that rejection of homografts is effected via the mechanism of an actively acquired immunity. Medawar postulated that this immunity response is developed by the host in order to reject genetically unrelated tissues, that the host's acquisition of homograft sensitivity is an immunological reaction, and that the prolongation of homograft survival is a function of immunological tolerance.

Genetic aspects. The genetic aspects of homotransplantation have been studied intensively[4-6] during the last twenty years, one of the first studies being of tumor transplantation.[4,17] However, today's interest has shifted largely to normal tissues.

Snell[18] proposed that the chromosomal locus relating to transplantation be called histocompatibility genes (H and h). Gorer and Amos[19] referred to this locus as H-2,[20] where, according to Billingham,[21] some of the alleles, now considered to be less than 12, are determinants for the production of transplantation antigens. Hoecker (1956)[22] presented a thorough discussion of the differences in the effects of H genes on transplantation. Schultz[23] pointed out that the basis for the behavior of tissues on transplantation in the immunological and genetic phenomena seemed to be divided between two contradictory bodies of data. On the one hand, the rigorous "laws of transplantation" demand a common genetic constitution of host and implant if a graft is to take. Where successful transplantation is concerned, the antigenic constitution of tissue is related to specific genotype as directly as are the classic immunological blood groups. On the other hand, in contrast to this definite picture are the experiments in which tolerance is induced, histocompatibility barriers are

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overridden, and tumors are enhanced in their growth or adapted to their hosts. Similarly, when the cellular basis of antibody synthesis is considered, analogous problems in cell heredity appear.

Cellular aspects. Murphy[24] and Loeb[25] believed that a causal relationship existed between the appearance of cells of the lymphocyte series (histiocyte, monocyte, or lymphocyte) and the homograft reaction. Medawar,[26] Algire *et al.*,[27] and Billingham *et al.*[28] all have supported findings that skin homotransplants evoked an immune cellular reaction, predominantly of the lymphocytic and plasmocytic cell types. Cellular antigens such as those in whole blood,[29] epidermal cells,[30] and disrupted lymphocyte cells[31] have been shown to be effective in the induction of homograft sensitivity.

Cellular components also have been responsible for the induction of tolerance and the specific inhibition of homograft specificity. Billingham *et al.*[31,32] have produced tolerance of skin homografts by injecting into embryos (mice, chickens, rabbits, and rats) viable cells (whole blood, leukocytes, mixtures of spleen, kidney, liver, and testis).

Several investigators confirmed these observations,[34,35] and others agreed that the nucleated cells are primarily the agents responsible for the recipient state of induced tolerance; thus it was only a matter of time before the question was asked, "Why do nucleated cells cause the induction of tolerance, and what components of these nucleated cells are the agents actively responsible for this phenomenon?"

Immunochemical aspects. Billingham *et al.*,[36] recalling the successful utilization of injected nucleated cells for the induction of acquired tolerance, set out to define the laws and nature of transplantation antigens in such cells. Using isolated nuclei prepared from spleen, thymus, or kidney of two strains of inbred mice (A, CBA), the "exclusive" transplantation antigens were found to be isolated in the nucleus. Cytoplasmic concentrates prepared from kidney and liver were entirely inactive when tested for their power to induce homograft sensitivity. A curious side light[7] of the latter experimental test is the suggestion that, when active at all, the cytoplasmic concentrates appeared to diminish the intensity of the normally expected homograft reaction. Isolated nuclei were found to be not as potent, when compared with intact cells, in their capacity to induce homograft sensitivity.

When desoxyribonuclease (DNase) was incubated with isolated nuclei of the spleen, the capacity for inducing homograft sensitivity was either completely or almost completely abolished. From these studies Billingham *et al.*[37] considered that the antigens concerned in the induction of homograft sensitivities were deoxyribonucleic protein possessing antigenic and genetic specificity.

Haskova and Hrubesova,[38] utilizing an experimental design similar to that of Billingham, injected desoxyribonucleic acid (DNA) from spleen, liver, and thymus, to determine whether it was capable of inducing any effect on skin homograft sensitivity. The results indicated that DNA by itself or coupled with traces of normal proteins is incapable of inducing skin homograft sensitivity. Billingham *et al.* now favor the belief that the weak activity of the preparations treated with DNase was due to contamination with antigenic impurities. He speculated that these impurities are probably polysaccharide in nature, this finding being analogous to his earlier finding that hexosamine contaminated highly purified preparations of DNA and that the nucleoprotein or the DNA may act as an adjuvant in maintaining the antigen in an appropriate physical form.

Kandutsch and Reinert-Wenck[39] have suggested that the antigen is in the form of a carbohydrate protein complex. They also have reported that the antigens probably are similar to blood group mucoids, perhaps amino acid polysaccharide complexes. Billingham *et al.*[37] now are of the opinion that polysaccharide or desoxynucleoprotein-polysaccharides are the responsible homograft antigens. Since it is clear that the nucleolus and the nuclei actively synthesize most of the cytoplasmic ribonucleic acid (RNA) and that this substance serves as a template for the synthesis of proteins and complex polysaccharides, it seemed important to determine the role of this type of nucleic acid in homotransplantation immunity. In an earlier paper we demonstrated that RNA isolated by the phenol method induced homograft tolerance when injected into pregnant rats. Evidence is presented in this paper to show that tolerance can be induced by pooled RNA derived from the skin, thymus, and spleen of donor rats.

Methods and Materials

Closed, randomly inbred rat colonies of the Long Evans (LE) and Wistar (W) strains were used in the following group of experiments. The RNA was taken from two sources: the skin of neonatal LE rats (LESRNA), and the spleen, liver, and thymus of adult LE rats (LERNA). At the time specified for each experimental group, the animals were switch-grafted by the Michel clip method.[40] The W animals were injected intraperitoneally; none of the LE rats was injected (FIGURE 1).

Organ RNA. Frozen liver, spleen, and thymus tissues from adult LE rats were minced and suspended (10% w/v) in saline-citrate buffer at *p*H 7.0). After the tissues were monogenized, the RNA was prepared by a method previously described.[40] The resultant preparation was checked for protein by the biuret test in approximately 1.0 ml. of solution. Under the conditions of the test, which is sensitive to 0.02 mg. protein, no protein was detected. RNA was determined by nitrogen:phosphorus ratios, which would also detect protein contamination, and by ultraviolet absorption. There was a slight variation in concentration from one preparation to another, but the average concentration was 0.37 mg. per milliliter.

Skin RNA (SRNA). Skin was removed from LE rats several days after birth and promptly frozen. The frozen skin was weighed and minced, then suspended (10% w/v) in saline-citrate buffer and homogenized. RNA was prepared from this homogenate, as in previous experiments. Since the skin was not as completely homogenized as the organ tissues, RNA concentrations were lower; the average concentration was 0.26 mg. per milliliter. The absence of protein was checked by the biuret test.

Treatment of RNA with ribonuclease. Crystalline ribonuclease (RNase) from beef pancreas was obtained from Mann Research Laboratories, New York,

N. Y. It had an activity of 38 Kunitz units per milligram; a 5-mg. quantity of this preparation was dissolved in 5 ml. of acetate buffer at *p*H 4.8. This solution was added to 120 ml. of an RNA preparation from the skin of newborn LE rats, and the solution was adjusted to a *p*H of 7.5. After an incubation period of 2 hours at 37° C. an equal volume of water-saturated phenol was added to remove the protein. The aqueous phase was extracted four times with ether, and the ether was removed by bubbling nitrogen gas through the solution.

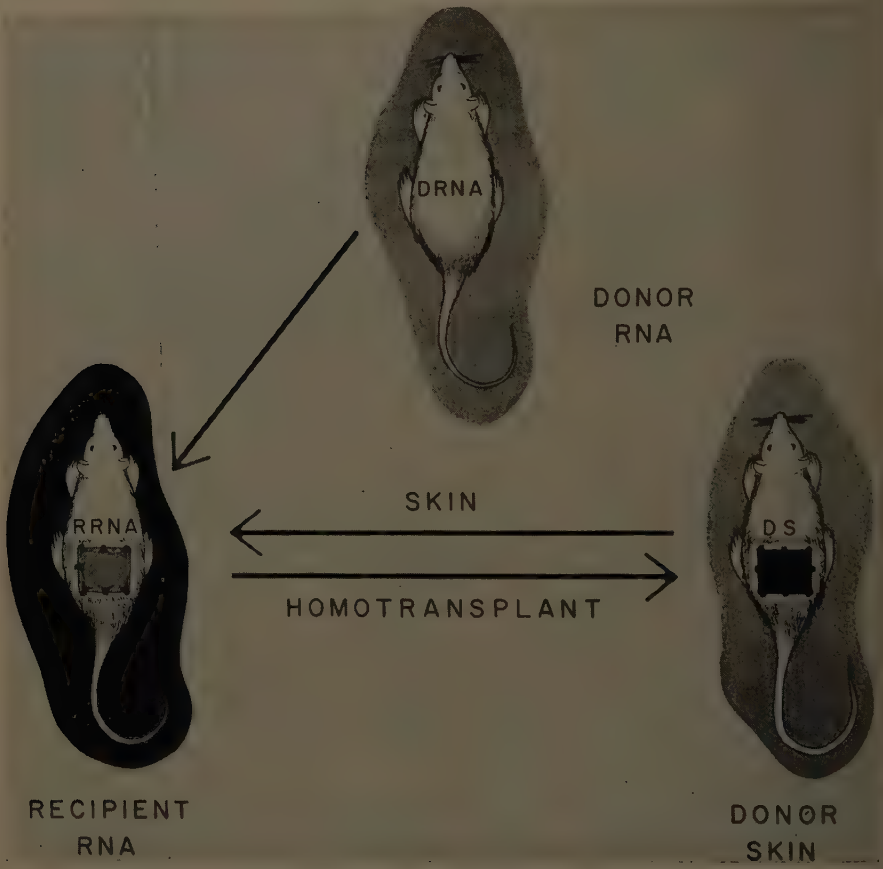

FIGURE 1. Steps in procedure of switch-grafting donor and recipient animals.

Group A (*0.1 cc. RNA*, *22-day series*). WRNA: 46 neonatal W rats were injected with 0.1 cc. of RNA (0.037 mg.) derived from organs of LE rats, and were switch-grafted at 22 days of age with 46 22-day-old LE rats (LERNA).

WC: 26 neonatal W controls (WC) were injected with 0.1 cc. of saline and were switch-grafted at 22 days of age with 26 22-day-old LE rats (LEC).

Group B (*0.1 cc. SRNA*, *22-day series*). WSRNA: 18 neonatal W rats were injected with 0.1 cc. (0.026 mg.) of SRNA and were switch-grafted at 22 days of age with 18 22-day-old LE rats (LESRNA).

WC: Controls from group A were used for group B.

Group C (0.4 cc. SRNA, 22-day series). WSRNA: 22 neonatal W rats were injected with 0.4 cc. (0.10 mg.) of SRNA and were switch-grafted at 22 days of age with 22 22-day-old LE rats (LESRNA).

WC: 25 neonatal W rats were injected with 0.4 cc. of saline and at 22 days of age were switch-grafted with 25 22-day-old LE rats (LEC).

Group D (0.5 cc. SRNA, 40-day series). WSRNA: 32 36-day-old W rats were injected with 0.5 cc. of SRNA (0.10 mg.) and were switch-grafted at 40 days of age with 32 40-day-old neonatal LE rats (LESRNA).

WC: 24 36-day-old W rats were injected with 0.5 cc. of saline and were switch-grafted when the animals were 40 days old with 24 LE rats (LEC).

Group E (RNase hydrolyzed RNA control (T), 40-day series). WT: 14 36-day-old W rats were injected with 0.4 cc. of RNase-treated RNA, and when the animals were 40 days old they were switch-grafted with 14 LE rats (LE-T).

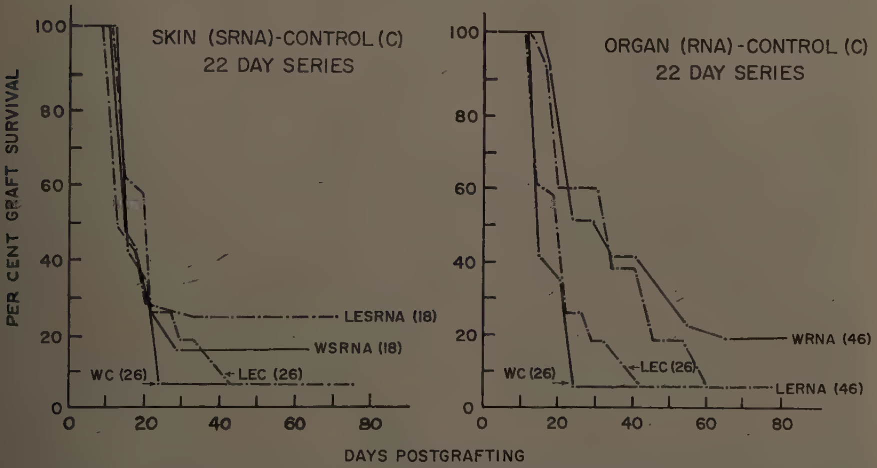

FIGURE 2. Percentage graft survival in WRNA and LERNA (*right*), in WSRNA and LESRNA (*left*), and in controls. The number of animals in each group is given in parentheses.

Gross observation and histological methods were used to evaluate the experimental groupings. The histological evaluation will be presented in a forthcoming publication.

Results

Group A (0.1 cc. RNA, 22-day series). Forty per cent of the WRNA grafts were viable for 45 days postgrafting; at 70 days postgraft 20 per cent were viable; 40 per cent of the LERNA grafts were viable 35 days postgraft, while only 8 per cent were viable 70 days postgraft.

Sixty per cent of the controls had sloughed by the twentieth postoperative day, and 8 per cent were viable at 60 days postgraft (FIGURES 2 and 3*a*).

Group B (0.1 cc. SRNA, 22-day series). Sixty per cent of both the WSRNA and LESRNA grafts had sloughed by postoperative day 19. At postoperative

60, 17 per cent of the WSRNA and 22 per cent of the LESRNA grafts were observed to be viable (FIGURES 2 and 3*b*).

Group C (0.4 cc. SRNA, 22-day series). Sixty per cent of the WSRNA grafts had sloughed by postoperative day 20, while 22 per cent were viable on postoperative day 60. Forty per cent of the LESRNA grafts were observed to be sloughed on postoperative 30; 33 per cent were viable on postoperative day 30. Sixty per cent of the control WC grafts sloughed by postoperative day 21; 8 per cent were viable on postoperative day 60. Forty percent of the LEC

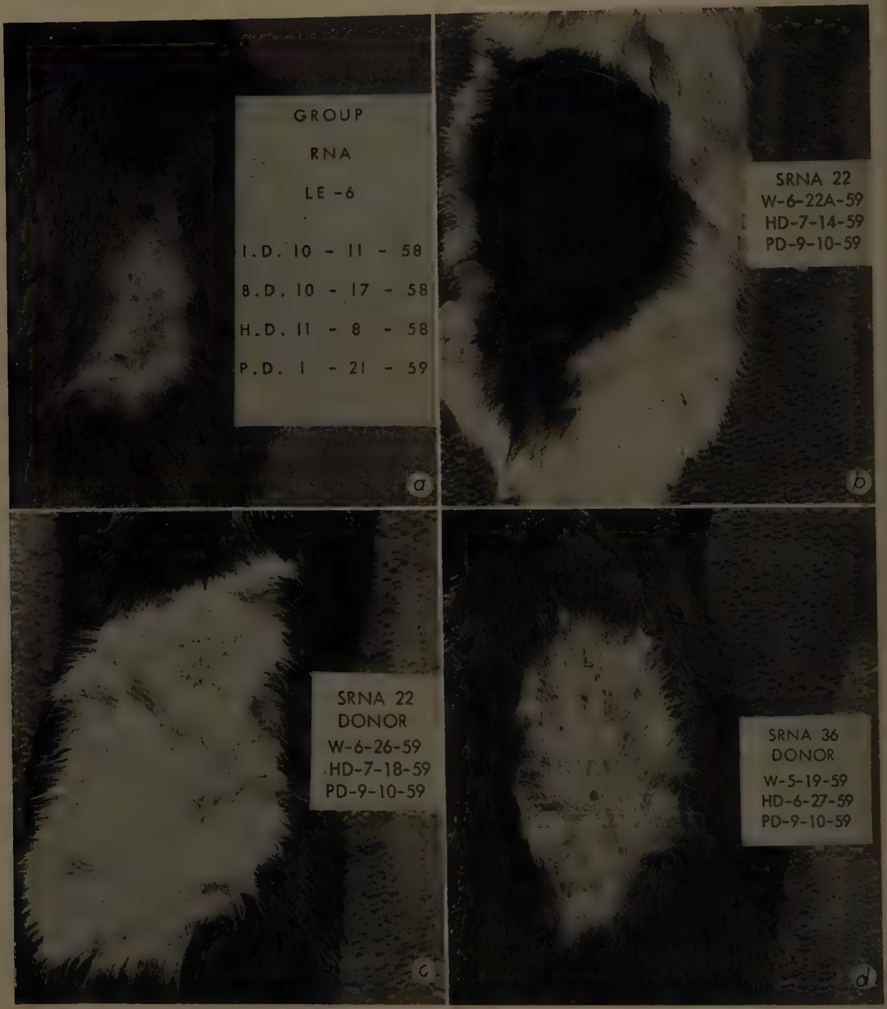

FIGURE 3. (*a*) Viable skin homograft on Long-Evans rat at 70 days (organal source of RNA). (*b*) Viable graft on Wistar rat at 56 days (skin source of RNA). (*c*) Viable graft on Long-Evans rat at 52 days (skin source of RNA). (*d*) Viable graft on Long-Evans rat at 70 days (skin source of RNA).

controls were viable on postoperative day 18, and 8 per cent were viable on day 40 (FIGURES 3*c*, 4).

Group D (*0.5 cc. SRNA, 40-day series*). By postoperative day 25, the WSRNA grafts had sloughed completely. However, the 40 per cent LESRNA grafts were viable on postoperative day 25; 20 per cent were viable at day 60.

All the control WC grafts were sloughed by postoperative day 20. Sixty per cent of the LEC grafts had sloughed by the postoperative day 19; 7 per cent were viable at day 60 (FIGURE 5).

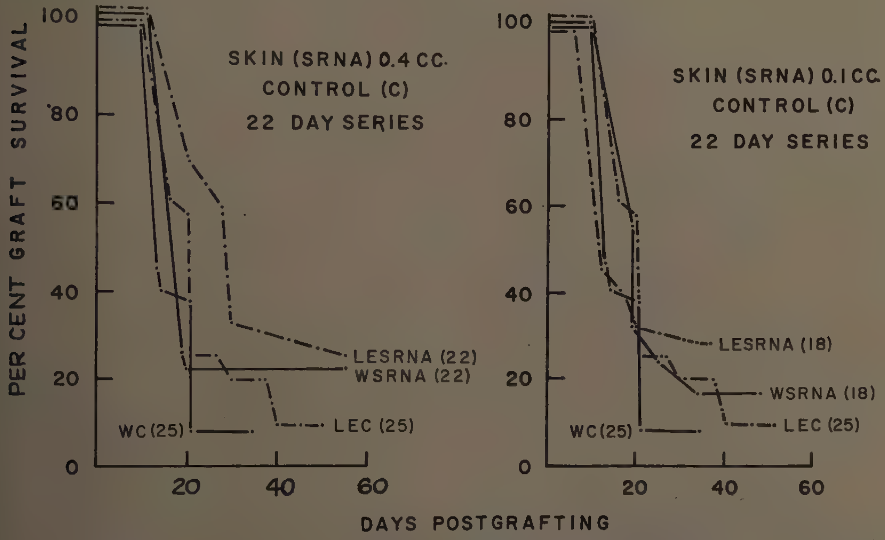

FIGURE 4. Percentage graft survival in WSRNA and LESRNA with 0.1 cc. injected (*right*), and 0.4 cc. injected (*left*). Controls are also included. The number of animals in each group is in parentheses.

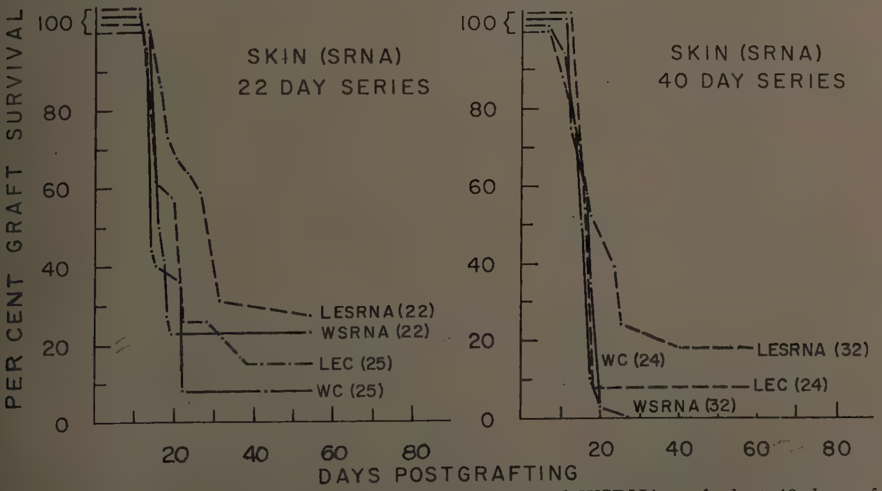

FIGURE 5. Percentage graft survival in LESRNA and WSRNA grafted at 40 days of age (*right*), and at 22 days of age (*left*).

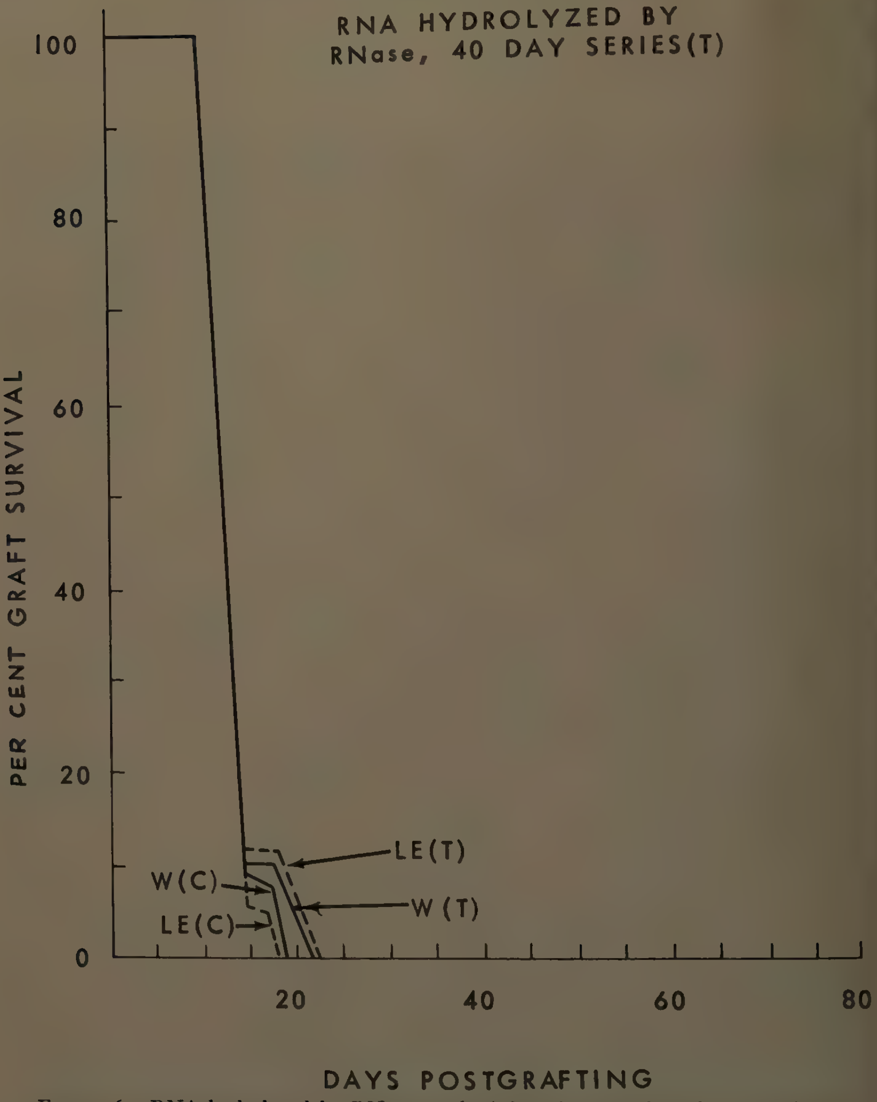

FIGURE 6. RNA hydrolyzed by RNase as the injected material, with appropriate controls.

Group E (RNase-hydrolyzed RNA control (T) 90-day series). LE-W grafts were nonviable on postoperative day 22. All of the LE and W controls were nonviable by postoperative day 19 (FIGURE 6).

Discussion

Most biochemists are of the opinion that cytoplasmic RNA is synthesized largely in the nucleus and nucleolus rather than in the cytoplasm.[41] Hence the observation made by Billingham and his co-workers,[37] that the nuclear preparations are more active as transplantation antigens than as cytoplasm, is consistent in part with our findings. Unless RNase inhibitors are added during the preparation of cytoplasmic RNA, degradation by the soluble RNases present in the cytoplasm would be expected, since cytoplasmic RNA appears to be more vulnerable to attack by these enzymes than is nuclear RNA. RNases are inhibited strongly in the presence of DNA and protein. RNA coated with protein is not available to RNase and should not be as labile as purified RNA. RNA viruses also are known to be resistant to pancreatic RNase unless the protein coat is first removed. DNases, on the other hand, degrade DNA, and a corresponding decrease of RNA synthesis should be the result.

Since, as numerous investigators have shown, purified nucleic acid prepared from a wide variety of sources is not antigenic, it is very unlikely that the purified RNA used in our experiments is acting directly as an antigen. The analogy that may aptly apply here in the case of donor RNA is that it is involved directly in the biosynthesis of donor antigens when injected into recipient animals, in much the same way that infective RNA viruses synthesize their own proteins during the course of replication in the infected cells.[42] Viral RNA prepared by the phenol-extraction method is protein-free and, as demonstrated by numerous investigators, is infective and replicates intact virus in a variety of host tissues.[43] Protein synthesis of the coat occurs under these conditions, and such protein may be demonstrated in the virus particles purified from tissues originally infected with viral RNA.

Further instances of protein synthesis induced by RNA have been reported by Niu and Twitty[44] and Niu,[45,46] who demonstrated that the organizer and other embryonic tissues of urodeles, when explanted into hanging drops of a balanced salt solution, released diffusible substances capable of initiating differentiation of the young gastrula ectoderm. The results of analysis of the medium withdrawn from such isolated cultures suggested that the active component was RNA. Studies with RNA isolated from mammalian organs have produced evidence of its ability to cause differentiation and of its possessing inductive specificity. When presumptive ectodermal cells were cultured in the presence of RNA, variations in the pattern of differentiation occurred, which presumably required specific protein synthesis by the recipient cells. For example, cultures treated with RNA from calf kidney induced the appearance of tubulelike condensations in urodele explants.[44-46] Since the only known role of RNA is that of a template in the synthesis of protein or complex polysaccharides, it is likely that such is the case in the experiments reported by Niu.

It was reported recently that RNA protein prepared from lymphoid tissue of animals previously immunized against a Gram-negative organism induced the synthesis of antibodies when injected into nonimmunized immature animals.[47] The transfer of antibody synthesis therefore may be accomplished by the injection of lymph node RNA from immunized animals, and represents a further demonstration of RNA's inducing the synthesis of specific donor proteins in recipient animals.

Oth and Castermans[48] demonstrated clearly that DNA protein or DNA per se in isolated nuclei, after the removal of RNA protein from these nuclear preparations, was completely unable to enhance homotransplantation immunity. Furthermore, fractions containing hexoses were toxic and did not induce immunity. In the light of the latter observations it seems unlikely that nuclear polysaccharides or DNA protein act as transplantation antigens. Soluble RNA protein preparations obtained from nuclei lacking detectable quantities of either DNA or polysaccharides contained all of the transplantation antigenic activity, and this activity correlated well with the concentration of RNA from these nuclear preparations. RNA prepared by the use of detergent from RNA protein complexes did not induce immunity in those experiments.[48] Hall and Doty[49] reported that the phenol method of extraction of RNA, in contrast to detergent methods, preserves much of the secondary structure of the RNA that existed in the RNA particle. Lysozyme decreased the activity of these preparations, but no hexoses could be detected in the supernatant following its use; moreover, there was no evidence of polysaccharide in the RNA protein fractions treated with lysozyme.[48] On this point our data and those of Kandutsch and Reinert-Wenck[39] seem to indicate that this basically charged enzyme is reversibly absorbed on the RNA and that the inhibition induced by lysozyme disappeared after its physical removal from active preparations.

It is clear that much of the RNA injected into the recipient was lost through hydrolysis by RNases in blood, body fluids, and cells. We have experiments in progress in which the RNA is coated prior to injection with basic peptides from unrelated sources. We hope that by this method the loss of activity mediated through RNases may be minimized.

In our studies controls of special types were necessary to show that tolerance was induced specifically by RNA. A sample of RNA, previously active in inducing homotransplantation tolerance, was treated with RNase. The RNase-treated RNA preparation did not induce homotransplantation tolerance. This experiment therefore rules out the possibility that polysaccharides, which sometimes contaminate impure RNA preparations, were responsible for tolerance.

RNA derived from rats unrelated to either the donor or the recipient strains did not induce tolerance (unpublished observations). Clearly, the tolerance we have observed is related to the specificity of the donor antigens produced in response to the injected RNA. When the quantity of RNA injected was increased, a corresponding increase was noted in homotransplantation tolerance.

It is obvious from the evidence available that all degrees of immunological paralysis are possible if sufficient antigen is present to depress antibody synthesis. The final extent and the period of homograft tolerance apparently are related to the effectiveness of the inhibition of antibody synthesis. We have unexpectedly observed homograft acceptance in the donor animals grafted with skin of the RNA recipients, a phenomenon that is difficult to explain. If the RNA recipient is synthesizing antibodies against the donor, its skin may have been exposed to recipient antibodies. This phenomenon is most marked, as this hypothesis would lead one to expect, when newborn rats are injected with small amounts of RNA and are grafted at 22 days or when immunologically mature rats (aged 36 days) are so treated. Under either condition immune paralysis may not be sufficiently complete to prevent antibody synthesis, and immunoselection may play a significant role. The question of immunoselection has been examined before by Barrett and Deringer,[50] who found that tumors, after being passed through F_1 hybrids from their strain or origin to some other, will undergo a change in frequency of homograft acceptance when tested in the F_2 progenies or in back-crosses of the two strains. Moreover, in short-term experiments Algire[51] and others[52-54] were able to show that these effects could be mediated by "humoral factors" and did not require cell-to-cell contact.

Another possible but less appealing explanation of tolerance in the donor animals may be advance. If the donor RNA induces the synthesis of donor antigen in the skin and if circulating antibody is not able to react in the cells containing these donor proteins, the skin thus may be so conditioned as to allow homograft acceptance through the persistence of donor proteins in such skin. The answer to this puzzling problem must await further experimentation.

The extent of homotransplantation tolerance following RNA treatment of recipient animals would be expected to be a function of several variables: quantity and quality of antigens synthesized, the range of genetic difference represented in the donor RNA pool, the route of administration, the time factors relating to the period and extent of paralysis, and the possible effect of physical and chemical alteration of RNA during the process of purification. Preliminary studies suggest that a single neonatal RNA injection and subsequent grafting at 22 days may be too long an interval for maximal tolerance. RNases are widespread in the animal body, and their effect must be considered.

At the present time we do not know what cells "infected" with donor RNA are capable of synthesizing donor proteins.

Summary

RNA derived from both the skin and various visceral organs of Long-Evans rats induced homograft tolerance when injected into recipient Wistars prior to the transplant of skin. Liver, spleen, and thymus RNA was quantitatively more effective than skin RNA. When control animals were injected with RNase-hydrolyzed RNA, it was observed that the treated RNA was unable to induce tolerance. Furthermore, RNA derived from rats unrelated to either donor or recipient was unable to induce tolerance. Homograft tolerance was induced in both donor and recipient animals to varying extents. Explanations of this unexpected phenomenon in the donor animals are discussed.

References

1. Fomon, S. 1939. *In* The Surgery of Injury and Plastic Repair. : 95–105. Williams & Wilkins. Baltimore, Md.
2. Medawar, P. B. 1956–1957. Harvey Lectures. **52:** 144. Academic Press. New York, N. Y.

3. Medawar, P. B. 1959. *In* Cellular and Humoral Aspects of the Hypersensitive States. : 504. H. S. Lawrence, Ed. Hoeber. New York, N. Y.
4. Brent, L. 1958. *In* Progress in Allergy. **V**: 271. S. Karger. New York, N. Y.
5. Gorer, P. A. 1956. *In* Advances in Cancer Research. : 149. Academic Press. New York, N. Y.
6. Snell, G. D. 1957. Ann. Rev. Microbiol. **11:** 439.
7. Lawrence, H. S. 1959. Physiol. Rev. **39:** 811.
8. Merrill, J. P. 1959. Physiol. Rev. **39:** 860–884.
9. Jacob, S. W., D. Gowing & J. E. Dunphy. 1959. Am. J. Surg. **98:** 55–90.
10. Cannon, J. A., P. I. Teraski & W. P. Longmire, Jr. 1957. Am. J. Surg. **94:** 303.
11. Longmire, W. P. Jr., 1953. J. Natl. Cancer Inst. **14:** 669–681.
12. Egdahl, R. H. 1958. Transplantation Bull. **5:** 87–97.
13. Conway, H. & R. B. Stark. 1954. Surgery. **38:** 487–518.
14. Chase, M. W. 1959. Ann. Rev. Microbiol. **13:** 349.
15. Rogers, B. O. 1951. Plastic and Reconstructive Surg. **7:** 169–202.
16. Medawar, P. B. 1945. J. Anat. **79:** 157.
17. Hauschka, T. 1952. Cancer Research. **12:** 615–653.
18. Snell, G. D. 1951. J. Genet. **49:** 87–108.
19. Gorer, P. A. & D. B. Amos. 1956. Cancer Research. **16:** 338.
20. Gorer, P. A. 1956. *In* Advances in Cancer Research. : 199. Academic Press. New York, N. Y.
21. Billingham, R. E. 1959. Science. **130:** 947–953.
22. Hoecker, G. 1956. Genetic Mechanisms in Tissue Transplantation in the Mouse. Cold Spring Harbor Symposia on Quant. Biol. **21:** 355–362.
23. Schultz, J. 1959. Science. **129:** 937–943.
24. Murphy, J. B. 1926. The Lymphocyte in Resistance to Tissue Grafting, Malignant Disease, and Tuberculosis Infection. Monogr. Rockefeller Inst. Med. Research. No. 21.
25. Loeb, L. 1930. Physiol. Rev. **10:** 547.
26. Medawar, P. B. 1944. J. Anat. **78:** 176.
27. Algire, E. H., J. M. Weaver & R. T. Prehn. 1955. J. Natl. Cancer Inst. **15:** 1737–1767.
28. Billingham, R. E., L. Brent & P. B. Medawar. 1954. Proc. Roy. Soc. London. **B143:** 53–80.
29. Medawar, P. B. 1946. Brit. J. Exptl. Pathol. **27:** 15.
30. Billingham, R. E. & E. M. Sparrow. 1955. J. Embryol. Exptl. Morphol. **3:** 265.
31. Brent, L. 1958. *In* Progress in Allergy. **V**: 271. S. Karger. New York, N. Y.
32. Billingham, R. E., L. Brent & P. B. Medawar. 1955. Ann. N. Y. Acad. Sci. **59**(3): 409.
33. Billingham, R. E., L. Brent & P. B. Medawar. 1956. Phil. Trans. **B239:** 357.
34. Ashley, F. L., R. F. Sloan, A. N. Schwartz, Sr., G. Joseph, H. Stein & W. P. Longmire, Jr. 1958. Plastic and Reconstruction Surg. **22:** 462–470.
35. Cannon, J. A., P. I. Terasaki & W. P. Longmire, Jr. 1958. A.M.A. Arch. Surg. **76:** 769–773.
36. Billingham, R. E., L. Brent & P. B. Medawar. 1956. Nature. **178:** 514.
37. Billingham, R. E., L. Brent & P. B. Medawar. 1958. Transplantation Bull. **5:** 377.
38. Haskova, V. & M. Hrubesova. 1958. Nature. **182:** 61.
39. Kandutsch. A. A. & U. Reinert-Wenck. 1957. J. Exptl. Med. **105:** 125.
40. Ashley, F. L., E. G. Mc Nall, E. N. Garcia, R. F. Sloan & J. W. Edwards. 1959. Am. J. Surg. **98:** 217–222.
41. Brachet, J. 1957. Biochemical Cytology. Academic Press. New York, N. Y.
42. Gierer, A. & G. Schramm. 1956. Nature. **177:** 702.
43. Gierer, A. & G. Schramm. 1956. Z. Naturforsch. **11b:** 138.
44. Niu, M. C. & V. C. Twitty. 1953. Proc. Natl. Acad. Sci. **39:** 985.
45. Niu, M. C. 1958. Anat. Record. **131:** 585.
46. Niu, M. C. 1958. Proc. Natl. Acad. Sci. **44:** 1264.
47. Hrubesova, M., B. A. Askonas & J. H. Humphrey. 1959. Nature. **183:** 97.
48. Oth, A. & A. Castermans. 1959. Transplantation Bull. **6:** 418.
49. Hall, B. & P. Doty. 1959. J. Molecular Biol. **1:** 111.
50. Barrett, M. K. & M. Deringer. 1950. J. Natl. Cancer Inst. **11:** 51.
51. Algire, G. H. 1957. Ann. N. Y. Acad. Sci. **69**(4): 663.
52. Klein, E. & G. Klein. 1956. Transplantation Bull. **3:** 136.
53. Klein, E. & G. Klein. 1957. Symposia Soc. Exptl. Biol. **No. 11:** 305.
54. Klein, G. 1956. Ann. Rev. Physiol. **18:** 13.

Discussion of the Paper

BARRETT: What I have to comment upon is related directly to the contents of Ashley's paper but, fortunately, I wish to tie my comments in with some of the papers in this monograph that report upon the use of various cellular extracts for studying these phenomena.

I shall start by telling you what I am *not* saying. It would be foolish to say that cell extracts are not antigenic or that DNA or RNA proteins are not antigenic. I am not saying that these things are not antigenic, and that some of the observations reported were not accurately made. However, I do want to make the point that, at least in my particular view, sometimes in the experimental results that do take place the other way around, the paradoxes and the anomalies are more thought-provoking than the results that fall nicely into line. As briefly as I can, I propose to call your attention to something of which some of you are already aware but may not have thought of in this context. I have described in our paper in this publication a system that we use in our laboratory for the study of the phenomenon of tumor immunity, using the DBA/2 tumor in the BALB/c mouse as a recipient. I think that any one who saw the data that we have presented over a number of years recognizes that, whether this system is a special one, whether it is a sensitive one, or whether it is not a sensitive one, the data are, nevertheless, certainly very clear-cut. We are dealing with something that produces a high-grade tumor immunity and also a skin immunity.

This can be accomplished easily by using washed erythrocytes, washed erythrocyte stromata, and a great many other things. When we produce this immunity, several things that reflect upon not only Ashley's paper but the others are apparent.

Walter Schneider of the National Cancer Institute cooperated and, with his very delicate methods for studying DNA and DNA protein, he reported to me that our whole blood material had a small quantity of DNA in it. The separated leukocytes contained all the DNA that could be found in the whole blood, and neither the washed erythrocytes nor the stroma appeared to contain any DNA detectable by his very delicate methods. However—and I have not said that the DNA is not antigenic—I said that materials in our system in which it is unimaginable that there is any quantity of DNA or DNA protein are still highly antigenic. Accordingly, I point out that, to me, it is very thought-provoking that here is an antigen for tumor immunity that has no DNA in it. It has no DNA protein; it has no RNA protein.

On the other side of the picture relating to the extract, the erythrocytes were lysed; in each of the three lysing methods used, a piece of the cell membrane was preserved. The antigen was apparently undiminished in strength in each of the three gentle methods that broke the membrane up into pieces smaller than one micron. This is hardly putting anything into solution. The antigen was entirely gone, in so far as I could detect it in my system. The tumor used is highly antigenic, and it is easier to achieve a type of immunity with it than with the red blood cells. This tumor, obviously, contains a great deal of DNA protein and other elements that one would expect to find in a cell. The tumor was subjected to what I consider relatively gentle treatment:

treatment under which many antigens have survived many times over; it was subjected at 8.9 kc. in a sonicating machine at 2° C. for about 6 min. The red blood cells usually succumbed in 90 sec.

When the tumor material was then tested (by a method that I have not the space to describe) against an aliquot not put through the sonicating machine, it was found that the unbroken tumor cell was as highly antigenic as one would always expect, but that the sonicated tumor, which by Schneider's analysis contained the same amount of DNA protein as did the intact tumor, had no antigenicity in our experimental system.

Therefore, to return to where I started in my analogy: I do not say that DNA is not antigenic for transplantation immunity. I do not say that cell extracts are not antigenic in transplantation immunity; but I find it most thought-provoking that material that does not have any DNA protein in it and is not extractable by any imaginable method at the present time is still highly antigenic.

Koprowski: I have a few questions about the properties of the RNA used. First—and I did not understand what was done *in vitro* and what *in vivo*—have you subjected the RNA to tests that would indicate that this is an RNA?—tests such as action of ribonuclease? Second, is your preparation, for instance, resistant to high molar salt concentration?

The only other biologically active preparation of RNA known to me is the virus RNA. If you inject preparations of such RNA, known to be RNA, into animals, the RNA, in order to form the infectious virus, must find susceptible cells immediately, because the amount of ribonuclease in the body of an adult mouse (and I presume in a newborn mouse) is such that prompt inactivation occurs.

This, I think, is perhaps a point of weakness in your system. You have the postulate that the RNA persisted for some time in the body of the mouse in its intact form in order to produce the desired results. This is incompatible with what we know about virus RNA.

RNA does not cause formation of antibodies—we all agree to that. The antibodies you found must have been elicited by impurities in your preparation—such as, for instance, the donor protein.

I think it would be of great importance to elucidate these facts and to find out whether your preparation is really pure RNA. If the chemical tests yield conclusive results, you must find an explanation for persistent activity of the preparation after putting it in the body of an animal.

Norman Molomut (*Waldemar Medical Research Foundation, Port Washington, N. Y.*): A relationship to virus RNA and self-replication in only one cycle, demonstrable in our recent experiments, may shed some light on Ashley's and Barrett's findings.

We treated a single pure strain of mice (A-strain mice), with lyophylized tissue homogenates of both normal and cancer tissues from other strains. There were no whole cells here! Barrett may be interested in this, and I agree with him fully that the antigenicity of DNA and RNA are not in question, but I wonder whether it is necessary, as in his system, to have whole cells.

These A-strain mice who were treated with tissues from other strains were then implanted with an A-strain tumor, Sarcoma 1, from an untreated A-strain mouse. This tumor now proliferated in the treated mouse and was transplantable directly to the mice of the donor strains and also to two other strains whose genetic relationship to the A strain was sufficiently close to be a threshold relationship.

Here is a situation where the self-replication of the tumor cell from a small inoculum of the tumor graft, self replicated in the treated animal of its own strain, and appeared with properties that made it directly homotransplantable to at least three other strains of mice in repeated subpassages for more than seventy generations of passage in these strains and still retained its ability to regrow in A-strain mice for several generations.

D. STEINMULLER (*Wistar Institute*): Did the percentage of graft survivals in Ashley's curves refer to percentages of survival of individual grafts at various times or to proportions of surviving grafts in his sample? Could we, in terms of the importance of his conclusions, have some idea of the normal median survival time (MST) of his homografts and their ranges as compared to the MSTs of your treated recipients? Also, how many cases of donors accepting the recipient's tissue does he have to support the statement made in his paper about reciprocal tolerance?

ASHLEY: With respect to the characteristics of the RNA that we have prepared, we precipitate our RNA with salt under conditions that lead to almost no DNA contamination. We used nitrogen:phosphorus ratios that came within 2 per cent of theoretical values. Chromatography of the free bases showed that we had all four of the bases that would be expected, and in the same ratio that had been reported earlier for RNA preparations from rats.

The absorbancy in the ultraviolet was the absorbancy that is characteristic of RNA. In the experiment that we reported with RNase-treated RNA, we carried the hydrolysis to at least 80 to 90 per cent completion, and we lost all activity.

With regard to the rat studies and the controls: we have run quite a large number of controls, and we have controls within each group that we run. We ideally take a minimum of two animals from each litter as controls.

The number of "takes" that we obtain in our control groups relates entirely (and I must emphasize *entirely*) to the sizes of the grafts. When we use a small graft 2 cm. in diameter, we observe consistent sloughing.

With regard to Barrett's comment: of course, we have no information with regard to what our RNA is doing, beyond the knowledge that it certainly makes donor proteins. It may indeed be making donor polysaccharides, and our work certainly does not show that only RNA per se is of importance in conditioning animals or is the only active element. Precisely, what we are saying is the very opposite: we are saying that RNA can replicate many complicated molecules, so many that we cannot even make an intelligent guess as to which of these are of importance in the histocompatibility system.

In regard to the strong RNase effect in serum: this is a very good one in the serum and in blood of the recipient animal, and it was largely on this point that I was very much surprised to see that we could obtain any "takes" at all with the extremely low levels of RNA that we were using, for 0.37 mg./ml. is a very small amount, and we did not anticipate that we should achieve an effect with that small amount.

We have work under way in which we have coated the RNA with an unrelated peptin in order to attempt to escape the problem of an RNase effect on this polymer. There is no way, certainly, by which we can ascertain what percentage of this RNA, once in the cells, is happily replicating. I suspect that a very large percentage of it is destroyed.

THE WEIGHT-GAIN ASSAY FOR RUNT DISEASE IN MICE*

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A particularly violent and reproducible form of the reaction on the part of transferred lymphoid cells against a nonreacting host occurs following the intravenous infusion of a suitable dose of spleen cells of the DBA/1 strain of mice into neonatal C57BL/6 recipients. Therefore, we have confined the studies very briefly described in this preliminary report to the disease produced in this fashion and have made special use of the curve of weight gain of the injected neonatal animals as a rough indication of the course of the battle between graft and host. The descriptive name runt disease was devised by Billingham and Brent,[1] who first observed it in mice.

Although there are occasional individual exceptions, the normal progress of weight gain of the C57BL/6 mice we have used, expressed as a simple average figure and plotted against time, is quite reproducible as a linear relationship from an initial weight of 1.4 to 1.6 gm. at birth to about 12 gm. at the end of 30 days (FIGURE 1).

The Appearance of Runt Disease and the Effect of the Dosage of Spleen Cells

Our standard dose of DBA/1 spleen cells has been 1.5×10^6 prepared in approximately equal parts from male and female donors according to the method of Billingham *et al.*[2] and administered intravenously within 24 hours of birth in a volume of about 0.05 ml. of Hanks' solution by their technique.[1] Animals dying within 2 days of the injection are assumed to have succumbed to its direct mechanical consequences and have been excluded from further consideration.

No detectable effect appears in the surviving young C57BL/6 recipients until the fifth to eighth day. At this time (FIGURE 1) an abrupt cessation of weight gain occurs in all injected animals. A growth plateau then supervenes for a period of about 10 days in most instances before death inevitably occurs. During this plateau period the animals steadily become weaker, lose some hair, particularly around the eyes, nose, and anus, and may develop abdominal swelling with ascitic fluid. No diarrhea occurs. The distribution of the time of death of 39 newborn C57BL/6 mice injected with 1.5 to 2.0 million DBA/1 spleen cells is shown in FIGURE 2. Typical runt disease is regularly encountered, however, with spleen cell doses as small as 120,000 cells and, in the animals injected with this dose, the time of death was not delayed, indicating little advantage on the part of recipients of this

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small dose over animals receiving doses of about 2 million cells. Doses of even 50,000 cells have resulted in clear-cut changes of the kind referred to above, ending in death. Larger doses, up to 2.5 million cells, have not shortened the period of grace before the inevitable appearance of the signs of runt disease, nor has death been hastened.

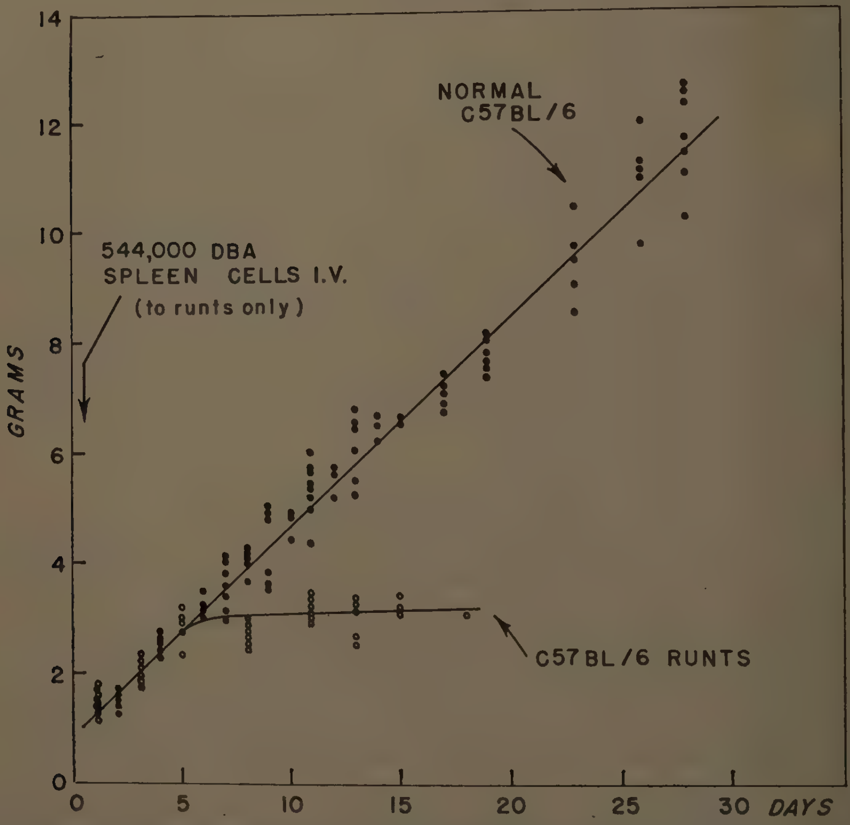

FIGURE 1. Weight gain curves for normal C57BL/6 mice and for a single C57BL/6 litter receiving 544,000 DBA/1 spleen cells intravenously within 1 day of birth. The normal curve is a composite of several litters, each animal being represented by a single solid point. Each of the injected animals is represented by an open circle.

Pathological examination of these runts during the plateau interval reveals profound atrophy of lymph nodes which, when they can be found at all, on microscopic examination are seen to be virtually replaced by large histiocytes. This finding is similar to that described by Gorer and Boyse in F_1 hybrid adult mice following spleen cell injections from parental strains.[3] The spleens were either slightly enlarged or normal in size in this fulminating and severe form of the disease and were histologically very much as described by Billingham and Brent[4] in that the normal Malpighian corpuscular architecture was usually indistinct in the face of a horde of large pale histiocytes, a finding emphasized by Gorer and Boyse.[3] Sparse collections of mononuclear cells are seen, particularly in the periportal areas of the moderately enlarged liver. The striking yellow patches along the free edges of the liver lobes, well described by Siskind and Thomas,[5] prove to be areas of liquefaction necrosis, with a variable population of surrounding leukocytes when viewed microscopically. Although the kidneys usually appear normal, there are some in which collections of mononuclear cells are seen in the subcapsular region. The skin appears to be entirely normal, both grossly and microscopically. Evidences of inflammatory changes in the lungs are rare.

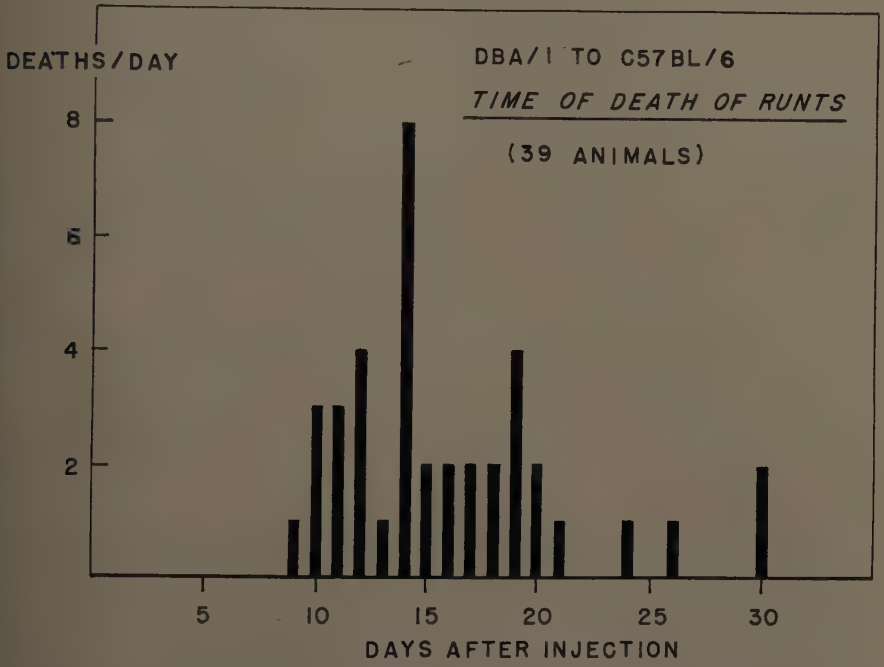

FIGURE 2. Time of death of runts. The time distribution of death of 39 C57BL/6 mice receiving from 1 to 2 million DBA/1 spleen cells intravenously within 1 day of birth.

Protection Against Runt Disease

The damaging effects of 1.5 million DBA/1 spleen cells can be abrogated either by immediately giving a second intravenous injection of an equal number of spleen cells from untreated adult C57BL/6 mice in an equal volume of Hanks' solution or by administering the same number of both types of cells as a mixture at one time.

Following either of these treatments, the weight gain curve of the injected neonatal animals is indistinguishable from that of untreated mice. Skin grafts from adult female members of the DBA/1 strain to 7 such doubly injected mice at 5 to 6 weeks of age were rejected in the normal fashion, with no indication of either tolerance or sensitization. Examination of these animals after sacrifice, however, often reveals distinctly small lymph nodes and slightly enlarged spleens. The livers are normal. If spleen cells from C57-BL/6 adults sensitized 2 to 3 weeks previously with DBA/1 skin grafts, and the intraperitoneal injection of 2 million spleen cells from DBA/1 mice are substituted for the untreated donors of the C57BL/6 spleen cells in the experiments just described, the results are the same, save that the minor pathological suggestions of runt disease in the survivors have not been detected.

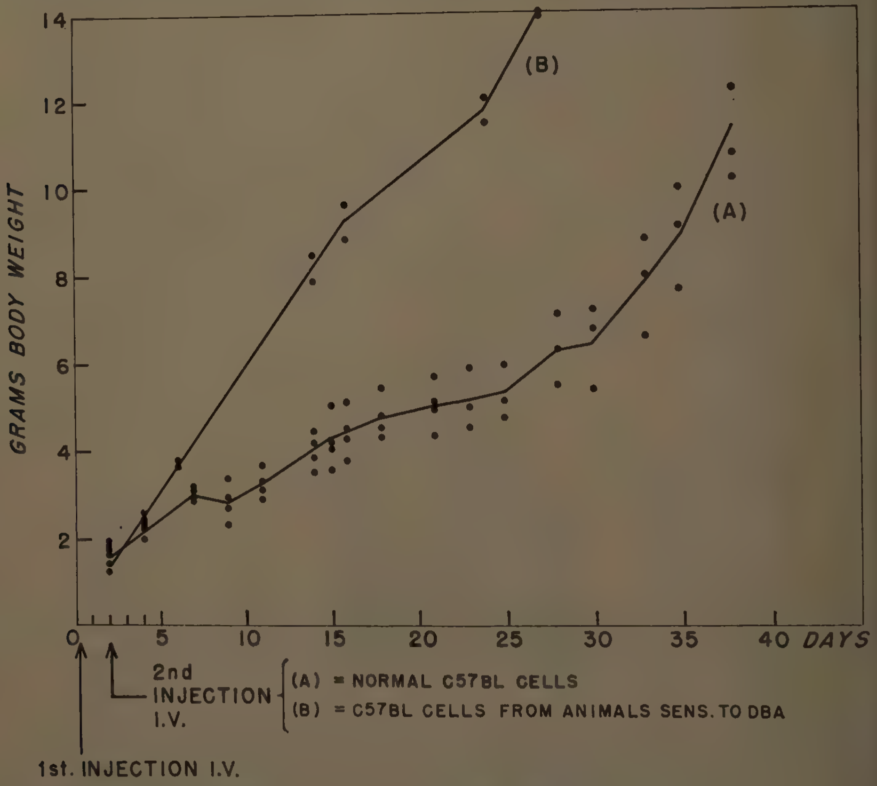

Figure 3. Re-equipment on the third day of life. Weight gain development of 2 groups of animals after intravenous injection of 1.5 million DBA/1 spleen cells within 1 day of birth. One group (A) has received a second injection of an equal dose of normal C57BL/6 spleen cells intravenously 2 days later; the other group (B) has received its second intravenous dose of C57BL/6 spleen cells from animals previously sensitized to DBA/1 mice (*see text*).

These results are in close agreement with the observations of Billingham and Brent[1] and with those of Siskind and Thomas.[5]

By delaying the protective intravenous injection of C57BL/6 spleen cells until 2 days after birth, a striking difference between the rate of action of cells from normal donors as against those previously sensitized to DBA/1 tissue is revealed.

Figure 3 shows the course of weight gain of 2 groups of potential runts, each treated by one of these 2 types of cells on the third day of life. The sensitized cell treatment allows normal growth when delivered at this age, whereas those injected with unsensitized cells are indistinguishable from untreated runts until the end of 1 month, when the second injection at last makes itself known and a delayed burst of weight gain, parallel to that of normal mice, occurs.

Thus far a few preliminary efforts to protect neonatal C57BL/6 mice from impending runt disease by means of later serum injections from C57BL/6

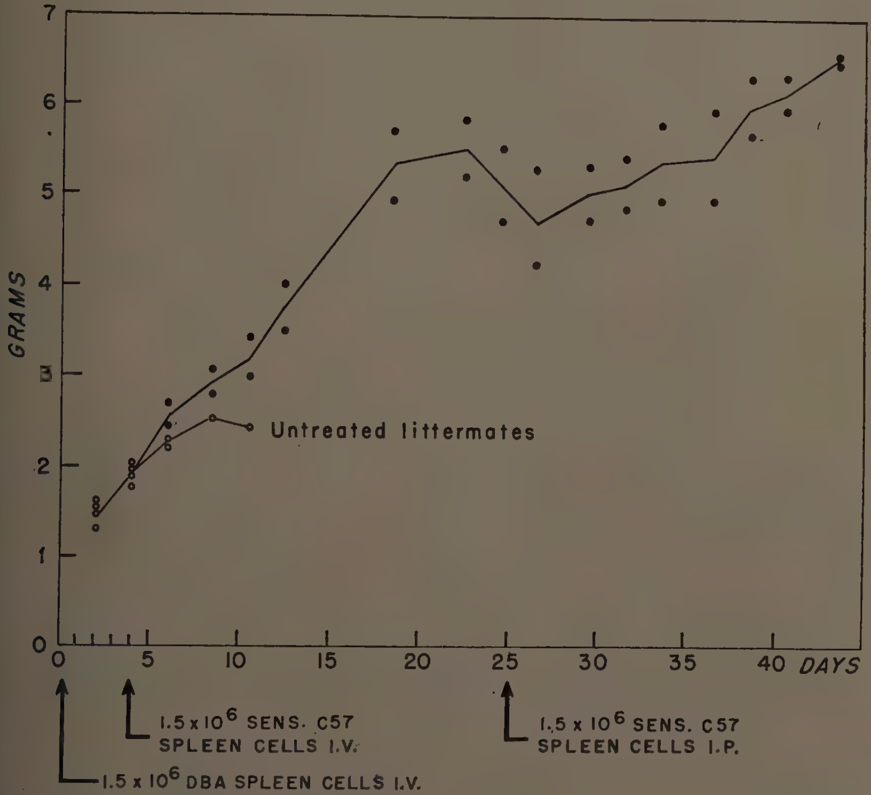

FIGURE 4. Re-equipment on the fifth day of life. The weight gain record of a single representative litter, each member of which received an initial injection of about 1.5 million DBA/1 spleen cells within 1 day of birth. Four days later one half of the animals were given a second injection of the same dose of C57BL/6 spleen cells from mice previously sensitized to DBA/1 tissues. A third injection of sensitized C57BL/6 cells was given at 25 days (*see text*).

adults previously sensitized to DBA/1 tissues, as described above, without the use of adjuvants, have been unsuccessful. This was an effort to confirm the interesting observation of Siskind and Thomas[5,6] that serum from animals sensitized with or without the use of adjuvants may protect against the later development of runt disease. Our experiments will be extended in this area.

If the second injection of spleen cells is further delayed to the fifth or sixth day of life, its protective effect is scarcely evident when unsensitized C57BL/6 cells are used. While this group of animals tends to live slightly longer than would be expected without treatment, its weight gain curve is the same as that of a group of untreated runts, and the difference in life span is probably not significant.

A second injection of about 1.5 million spleen cells from C57BL/6 mice sensitized to DBA/1 tissue as described above is still regularly effective at 4 days, however. In FIGURE 4 the course of a representative litter of this type is portrayed. Initially, there is a phase of progressive but subnormal growth that is soon dissipated, as a downward inflection of the weight-gain curve occurs at about 23 days. Another phase of growth can still be promoted at this time by a subsequent spleen cell injection intraperitoneally, indicating a delicate balance between the effects of the 2 types of cells. Intravenous injections after 5 or 6 days of age are technically difficult, because by this time the growing animals are rapidly developing their normal hair crop. Those whose survival is sufficiently long to allow skin grafting were all observed to react normally against adult DBA/1 female skin.

Attempts to treat runt disease after the plateau stage of weight gain has been reached have shown little success. At this stage only intraperitoneal injection is feasible. Even the injection of 5 million sensitized C57BL/6 spleen cells at 9 days resulted in no more than a brief period of subsequent weight gain in 4 animals before death resulted 2 to 10 days later.

Discussion

The pattern of development of runt disease, as measured by the course of weight gain of mice injected intravenously within the first day after birth, bears a close resemblance to the more familiar behavior of homografts in general. There is a period of apparent inactivity of the injected homologous cells during which weight gain is indistinguishable from that in untreated animals. The abrupt cessation of weight gain, after about 7 days, is reminiscent of the beginning of homograft rejection. It appears likely that it is this period of delay that permits the proliferation of as few as 50,000 spleen cells to bring about all the complex changes of this syndrome on approximately the same schedule as that observed with 2 million cells. Larger doses, within the range tested, appear to add little impact to the inoculum.

The salutory effect on the neonatal mice of an equal number of spleen cells from adult members of their strain does not depend on an *in vitro* mixture of such cells in advance of injection, since administration of the 2 types of cells by different veins without mixing is equally effective. Indeed, this effect may still come to bear on the second day, although it is less reliable unless the second dose of spleen cells is derived from an adult animal previously sensitized to animals isogenic with the primary spleen cell donors.

Further delay of the secondary injection of sensitized cells to 5 or 6 days can still effect a delay in the appearance of the first signs of runt disease. A later loss of weight usually occurs, however, under these circumstances, suggesting a delayed onset of runt disease. This, too, may respond to further treatment with sensitized spleen cells administered intraperitoneally.

The ineffectiveness of serum injections from sensitized animals in preventing homologous cells from causing runt disease is a preliminary finding and must be pursued further. An explanation of this difference from the results of Siskind and Thomas[6] may rest upon the fact that the cells that these investigators sought to influence by means of serum injections had been introduced intraperitoneally and possibly might have remained free-floating, and accordingly more vulnerable, for a longer time than our intravenously administered cells.

These findings, taken together, are fully consistent with the immunological explanation of runt disease as a graft versus host reaction, an opinion cogently advanced by Billingham.[7] Taking into consideration the indirect criterion of weight gain in these animals, it appears that in their earliest stages the pathological changes of this disease may be reversed. Once firmly established, the chain of events initiated by the injection of immunologically competent foreign cells appears to resist reversal by means aimed at destroying the injected cells.

I wish to express my gratitude to Elizabeth M. Sparrow, who contributed greatly to the beginning phases of this work, and to Mary L. Wood, for invaluable technical assistance.

References

1. Billingham, R. E. & L. Brent. 1957. A simple method of producing tolerance of skin homografts in mice. Transplantation. Bull. **4:** 67–71.
2. Billingham, R. E., L. Brent & P. B. Medawar. 1956. Quantitative studies on tissue transplantation immunity. III. Actively acquired tolerance. Phil. Trans. Roy. Soc. London. **B239:** 357–414.
3. Gorer, P. A. & E. A. Boyse. 1959. Pathological changes in F_1 hybrid mice following transplantation of spleen cells from donors of the parental strains. Immunology. **2:** 182–193.
4. Billingham, R. E. & L. Brent. 1959. Quantitative studies on tissue transplantation immunity. IV. Induction of tolerance in new born mice and studies on the phenomenon of runt disease. Phil Trans. Roy. Soc. **B242:** 439–477.
5. Siskind, G. W. & L. Thomas. 1959. Studies on the runting syndrome in newborn mice. J. Exptl. Med. **110:** 511–523.
6. Siskind, G. W. & L. Thomas. 1959. Studies on the runting syndrome in newborn mice. *In* Biological Problems of Grafting. : 176–185. Blackwell. Oxford, England.
7. Billingham, R. E. 1959. Reaction of grafts against their hosts. Science. **130:** 947–953.

THE RUNTING SYNDROME*

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Introduction

A number of workers, including Billingham and Brent,[1] Simonsen[2] and Woodruff and Sparrow,[3] have observed that, when newborn mice, rats, or chicks are treated with adult homologous lymphoid tissue, many of them will fail to grow at a normal rate and will die at two or three weeks of age. It has been reported by previous workers that these animals have diarrhea,[3,4] anemia with positive Coombs' test,[2] splenomegaly,[2,5,6] hepatomegaly,[5,6] and atrophic changes of their lymphoid tissue.[1,2,4,7] This disease has been referred to as the runting syndrome or homologous disease of newborn animals, and it is generally regarded as due to an immunological reaction of the homologous cells against a host that is unable to react against and destroy the foreign tissue.[1,2]

Experimental

Our work has been performed on newborn mice generally of A/Jax and DBA/2 inbred strains. Spleen cell donors are of the C57BL/6 strain. Spleen cell suspensions are prepared in saline solution, and the animals are injected intraperitoneally within 24 hours after birth. In most experiments each animal receives between 8 and 12 million homologous spleen cells. Death of the mouse at 5 to 30 days of age is an operational criterion of runting in most of these experiments.

As previously reported by ourselves[8,9] and by other workers,[2,4] runting requires that the donor cells be viable, immunologically competent, and of such genetic relationship to the recipient that they be able to react immunologically against the recipient. The incidence of runting depends upon the particular strain combination employed, increases as the dose of homologous spleen cells increases, and decreases as the time interval between birth and the administration of homologous spleen cells increases.

We found that runting in newborn mice receiving homologous spleen cells can be prevented by three techniques designed to enable the newborn animal to react immunologically against the foreign tissue.

Mice can be protected against runting by treatment with adult isologous spleen cells. Newborn BALB/c, CBA, and A/Jax mice receive 8 to 12 million C57BL/6 spleen cells intraperitoneally. Within one-half hour after receiving homologous spleen cells, one half of each litter is injected intraperitoneally either with 8 to 12 million isologous spleen cells or with isologous

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liver cells. As seen in TABLE 1, those animals that receive isologous spleen cells in addition to the homologous spleen cells have an incidence of runting of only 38 per cent as compared with 100 per cent for littermates receiving only homologous spleen. It may also be seen in TABLE 1 that isologous liver, given one-half hour after the homologous spleen, fails to alter the incidence of runting. When the isologous spleen injection is delayed to 1 day after the injection of homologous spleen, there is only minimal or no protective activity. In a second series of experiments, DBA/2 newborn mice are used as recipients and C57BL/6 mice are used as homologous spleen cell donors. Four groups of newborn mice are used. The first group receives 4 to 10 million homologous spleen cells, the second receives an equal number of isologous spleen cells, the third receives 4 to 10 million homologous spleen cells mixed with an equal number of isologous spleen cells, and the fourth is untreated. As shown in TABLE 2, the incidence of death among mice receiving homologous and isologous spleen cells is only 11 per cent, as compared with 83 per cent among mice receiving only homologous spleen. Both the untreated mice and the mice receiving isologous spleen cells have only a 10 per cent incidence of death.

TABLE 1

PROTECTION BY ADULT ISOLOGOUS SPLEEN CELLS AGAINST THE RUNTING SYNDROME*

Group	Incidence of death (No. dead/total no.)	
Newborn mice given homologous spleen	32/32	(100%)
Newborn mice given homologous spleen and isologous spleen 30 min. later	8/21	(38%)
Newborn mice given homologous spleen and isologous liver 30 min. later	20/20	(100%)
Newborn mice given homologous spleen and isologous spleen 1 day later	17/19	(95%)

* Recipients are A/Jax, CBA, and BALB/c mice. Homologous spleen cell donors are C57BL/6 adult mice. Each recipient receives 8 to 12 million homologous spleen cells.

TABLE 2

PROTECTION BY ADULT ISOLOGOUS SPLEEN CELLS AGAINST THE RUNTING SYNDROME*

Group	Incidence of death (No. dead/total no.)	
Newborn mice given homologous spleen	25/30	(83%)
Newborn mice given isologous spleen	2/21	(10%)
Newborn mice given homologous and isologous spleen simultaneously	4/35	(11%)
Untreated mice	2/21	(10%)

* Recipients are DBA/2 mice and donors are C57BL/6 mice. Recipients receive 4 to 10 million homologous or 4 to 10 million isologous spleen cells, or both, intraperitoneally within 24 hours after birth.

Mice can be protected against runting by treatment with serum from mice immunized against the spleen-cell donor strain. Newborn DBA/2 mice are treated with C57BL/6 spleen cells. Half of each litter then is treated for 8 days, starting 2 days after birth, with increasing doses of serum from DBA/2 mice that were immunized with C57BL/6 spleen cells. A total of 0.64 ml. of serum is given. Controls are treated with normal DBA/2 serum. As indicated in TABLE 3, the incidence of runting among mice receiving anti-C57BL/6 serum is 5 of 18, or 28 per cent, as compared with 14 of 15, or 93 per cent, among mice receiving normal serum.

TABLE 3
PROTECTION BY ANTISERUM AGAINST HOMOLOGOUS SPLEEN CELLS

DBA/2 litter no.	Incidence of runting (All received C57BL/6 spleen cells on day of birth) (No. runting/total no.)		
	Received normal DBA/2 serum	Received DBA/2 anti-C57BL/6 serum	No serum treatment
1	1/2	0/2	1/1
2	2/2	0/2	
3	1/1	1/2	
4	3/3	1/4	3/3
5	4/4	0/3	2/3
6	1/1	0/2	
7	2/2	3/3	
Total	14/15	5/18	6/7

TABLE 4
INCIDENCE OF RUNTING IN LITTERS FROM MOTHERS IMMUNIZED AGAINST HOMOLOGOUS SPLEEN CELLS

Treatment of mothers	Breeding cage no.	Incidence of runting (No. runting/total no.)
BALB/c mothers immunized against C57BL/6 spleen	1	11/12 (92%)
	2	3/12 (25%)
	3	2/10 (20%)
	4	9/10 (90%)
BALB/c mothers untreated	—	38/41 (93%)
A/Jax mothers immunized against C57BL/6 spleen	5, 6, 7	4/20 (20%)
A/Jax mothers untreated	—	78/79 (99%)

Mice can be protected against runting by immunizing the mother against the spleen cell donor strain. Adult female BALB/c mice are immunized by repeated intraperitoneal injections of C57BL/6 spleen suspensions. Adult female A/Jax mice are immunized by repeated injections into the toe pads of

C57BL/6 spleen cells in complete Freund's adjuvant. Offspring of these females and normal isologous males are injected with C57BL/6 spleen cells intraperitoneally within 24 hours after birth. As shown in TABLE 4, the incidence of runting is considerably less among the offspring of immunized mothers than among the offspring of normal mothers.

It is possible to transfer serially the runting syndrome; that is, newborn mice receiving spleen cells from isologous runts will themselves become runts. Spleens from 8- to 10-day-old DBA/2 runts that received C57BL/6 spleen at birth will cause runting of newborn DBA/2 mice. A/Jax mice are given 4 to 10 million CBA spleen cells at birth. Some of those surviving to 8 weeks of age are considerably smaller than normal A/Jax mice. These surviving runted mice are used as spleen donors. Recipients are newborn A/Jax mice. Each receives 4 to 10 million 8-week-old runted A/Jax spleen cells intraperitoneally. Fourteen of 19, or 74 per cent, of these recipients died. Thirteen of the deaths occurred between 6 and 10 days after birth. Among A/Jax mice receiving normal CBA spleen cells at birth a death rate of 22 of 39, or 56 per cent, was encountered, and only 3 of these deaths occurred before 15 days of age.

Discussion

Our findings are consistent with the hypothesis proposed by previous workers[1,2] that the runting syndrome is the result of an immunological reaction of homologous, immunologically competent cells against a host that is unable to react against and destroy the foreign cells.

The three methods by which we are able to protect against runting all represent ways of enabling the host to react against the foreign cells. Administration of adult isologous spleen cells might cause in the newborn animal an essentially "immunologically mature" state in which the animal is capable of reacting against and destroying the foreign tissue. Billingham *et al.*[10] have shown that isologous adult lymphoid tissue can eliminate skin graft tolerance. One may consider that the adult isologous spleen cells in our experiments acted in a similar manner to eliminate tolerance and enable the host to destroy the foreign cells. Considering the long time required for graft rejection after treatment of tolerant mice with adult isologous lymphoid tissue and the necessity that isologous cells be given soon after the homologous cells for protection against runting, it is likely that the mechanism of protection is related to the establishment of a state of "immunological maturity" and to the prevention of the establishment of a state of tolerance, rather than to the elimination of an established state of immunological tolerance.

The ability of immune serum to protect against runting provides indirect evidence of the possible importance of serum antibody in at least one step in the process by which an animal reacts against and destroys certain types of foreign tissues.

The reduced incidence of runting among offspring of mothers immunized against the spleen donor's strain is considered due to the passive transfer of antibody from mother to offspring, via either the placenta or the colostrum.

The ability of runt spleens to cause runting of isologous newborn mice is probably due to the persistence in the runted animal of immunologically active cells of the original donor's strain. The somewhat higher incidence of death and the earlier time of death observed among recipients of spleen cells from runted mice is reminiscent of a second-set or accelerated rejection reaction. Apparently, the foreign cells present in the donor runt have been in some way "sensitized" against the host's antigens and, when placed in a new host, give a more intense reaction against it, associated with a higher incidence of runting and earlier death among the recipients.

Summary

The runting syndrome, characterized by the failure of newborn animals to grow at a normal rate and by anemia, diarrhea, early death, splenomegaly, and focal coagulative necrotic lesions of the liver, can be produced by the intraperitoneal injection of homologous lymphoid tissue into newborn mice.

Mice receiving homologous spleen at birth can be protected from runting by early treatment with isologous adult spleen cells and by treatment with serum prepared from mice immunized against the spleen cell donor's strain. Offspring of mothers immunized against the spleen donor's strain exhibit a reduced incidence of runting.

Spleen cells from runted mice will cause runting of isologous newborn mice. The greater incidence of runting and earlier time of death in recipients of spleen from runted mice than in recipients of spleen from homologous mice are interpreted as a type of second-set or accelerated rejection phenomenon.

References

1. Billingham, R. E. & L. Brent. 1957. A simple method for inducing tolerance to skin homografts in mice. Transplantation Bull. **4:** 67.
2. Simonsen, M. 1957. The impact on the developing embryo and newborn animal of adult homologous cells. Acta Pathol. Microbiol. Scand. **40:** 480.
3. Woodruff, M. F. A. & M. Sparrow. 1957. Further observations on the induction of tolerance of skin homografts in rats. Transplantation Bull. **4:** 157.
4. Billingham, R. E. 1958. Studies of the reaction of injected homologous lymphoid tissue cells against the host. Ann. N. Y. Acad. Sci. **73**(3): 782.
5. Cock, A. G. & M. Simonsen. 1958. Immunological attack on newborn chickens by adult cells. Immunology. **1:** 103.
6. Simonsen, M., J. Engelbreth-Holm, E. Jensen & H. Poulsen. 1958. A study of the graft-versus-host reaction in transplantation to embryos, F1 hybrids and irradiated animals. Ann. N. Y. Acad. Sci. **73**(3): 834.
7. Egdahl, R. H., F. R. Roller & R. L. Varco. 1958. Acquired tolerance to homografts and heterografts in the rat. Ann. N. Y. Acad. Sci. **73**(3): 842.
8. Siskind, G. W. & L. Thomas. 1959. Conference on Biological Problems of Grafting. Liége, Belgium.
9. Siskind, G. W. & L. Thomas. 1959. Studies on the runting syndrome in newborn mice. J. Exptl. Med. **110:** 511.
10. Billingham, R. E., L. Brent & P. B. Medawar 1959. III. Quantitative studies on tissue transplantation immunity. Phil. Trans. Roy. Soc. London. **B239:** 357.

QUANTITATIVE STUDIES ON THE INDUCTION OF TOLERANCE OF HOMOLOGOUS TISSUES AND ON RUNT DISEASE IN THE RAT*

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INTRODUCTION

The principle that rats may be rendered tolerant of homologous tissue grafts following pre- or early postnatal exposure to living homologous cells is now well established (Woodruff and Simpson, 1955; Billingham *et al.*, 1956; Lengerová, 1957; Egdahl *et al.*, 1958). It is also known that "runt" disease (Billingham, 1958; Billingham and Brent, 1959) not infrequently occurs in this animal, as in the mouse, if the inoculum employed to induce tolerance contains a high proportion of immunologically competent cells (Woodruff and Sparrow, 1957; Egdahl *et al.*, 1958; Billingham and Silvers, 1959*a*).

Their size renders rats much more convenient subjects than mice for detailed analyses of quantitative aspects of the induction of tolerance and the pathogenesis of runt disease: for example, large amounts of bone marrow can be obtained from a single animal, thoracic ducts of rats can be cannulated to obtain almost pure lymphocytes in considerable quantities, and daily samples of blood may be taken from very young animals for exact hematological analyses without prejudicing their well-being. Unfortunately, however, these advantages hitherto have been more than counterbalanced by the lack of the necessary isogenic strains, so that present knowledge derives largely from work conducted on mice.

The recent availability of 2 highly inbred strains of rat, the Lewis (albino phenotype) and the B.N. (chocolate), differing so widely with respect to their origins that the degree of genetic disparity between them is likely to be very great (Billingham and Silvers, 1959*b*), has terminated this unsatisfactory state of affairs and thus facilitated the investigations that form the subject matter of this paper.

The fate of interstrain skin homografts. Destruction of skin homografts exchanged between adult members of the B.N. and Lewis strains is invariably complete within 13 days, and the median survival time (MST) of Lewis → B.N. homografts does not differ significantly from the reciprocal combination. However, it is of some interest that the MSTs of adult skin homografts transplanted to newborn recipients are strikingly different: Lewis → B.N., 19.3 days; B.N. → Lewis, only 12.6 days. The possible significance of this difference will be apparent later.

Preparative details for the different types of cell suspension used and for the skin-grafting procedures have been given in full elsewhere (Billingham

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and Brent, 1959; Billingham and Silvers, 1959a). Throughout this study all inoculations of cells into newborn rats were made directly into the blood stream via the orbital branch of the anterior facial vein to ensure maximal rate and completeness of dissemination into the host's tissues and organs, a necessary condition for optimal results with respect to the induction of tolerance and of runt disease (*see* Billingham and Brent, 1959; Billingham and Silvers, 1959*a*). For experiments in which the induction of tolerance of skin homografts from the donor strain was the objective, the neonatal animals received suspensions of bone marrow cells throughout to minimize the possibility of causing runt disease, since this tissue contains relatively fewer immunologically competent cells than any other practicable source of living tissue cell suspension.

TABLE 1
INDUCTION OF TOLERANCE BY INTRAVENOUS INJECTION OF VERY YOUNG RATS WITH HOMOLOGOUS BONE MARROW CELLS

Strain combination	Dosage of cells injected (millions)	Age of hosts at injection	No. test-grafted	Per cent tolerant	Per cent highly tolerant*
Lewis → B.N.	2.5	24 hours	8	12.5	0
	5.0	24 hours	22	32.0	26.0
	7.0	24 hours	18	94.0	72.0
	15.0	24 hours	15	100.0	93.0
	15.0	3 to 4 days	28	43.0	11.0
	15.0	5 days	11	27.0	0
B.N. → Lewis	5.0	24 hours	13	0	0
	10.0	24 hours	16	31.0	0
	20.0	24 hours	21	33.0	10.0
	40.0	24 hours	17	100.0	?

* Highly tolerant animals are those that accepted their test skin homografts for longer than 50 days.

RESULTS

Cell Dosage in Relation to the Induction of Tolerance and Duration of the Tolerance-Responsive Period

The results (TABLE 1) show that for both donor/recipient combinations tested tolerance of subsequent skin homografts transplanted in adult life can be induced by neonatal inoculation with homologous marrow cells. With the Lewis → B.N. combination, however, this procedure confers tolerance on a much greater proportion of the animals.

Cell dosage is clearly a much more important factor in the induction of tolerance than hitherto suspected. For example, with the Lewis → B.N. combination, increasing the dosage of marrow cells injected into newborn hosts from 5 to 7 million cells almost trebled the proportion that became tolerant. Similar evidence as to the importance of this dosage factor was also obtained with the B.N. → Lewis combination. This sharp rise of the cell-dosage/tolerance response curve is interesting in view of the rather feeble effect of dosage when adult animals are *sensitized* with orthotopic grafts of homologous skin or with suspensions of homologous cells inoculated parenterally (Medawar, 1957; Billingham *et al.*, 1957). The most probable explanation of the great importance of dosage in the one situation and its relative insignificance in the other is that the *sine qua non* for the induction of tolerance is that all maturing cellular ingredients of the immunological response machinery of a young animal must be thoroughly exposed to the cellular antigen concerned to induce the complete central inhibition of response underlying tolerance. In the adult animal, however, exposure of only a part of its lymphoid tissue system to the antigen will elicit a sensitization that can be expressed systemically.

Although the data bearing upon the duration of the tolerance-responsive periods for the 2 strain combinations (TABLE 1) are as yet incomplete, it may be concluded safely that the Lewis rat at birth is already nearing the end of its tolerance-responsiveness to B.N. cells. B.N. rats, on the other hand, still retain a high degree of tolerance-responsiveness towards Lewis cells as late as the third day postpartum, although even here the decline of this immunologically interesting phase of their life span is far advanced by the fifth day.

In the light of our findings as to the importance of the dosage factor, it is clear that the influence of inoculation of much higher dosages of donor marrow cells into the older hosts must be explored fully before definition of the 2 tolerance-responsive periods under investigation is complete. In Woodruff and Simpson's (1955) experiments, where the influence of dosage per se was not investigated in relation to the timing of inoculation, the decision to increase the inoculum given the older and larger recipients probably had an important bearing on the results.

Runt Disease: Clinical Picture and Pathology

In very young mice natural infections and diseases may produce overt symptoms, such as diarrhea and retardation of growth, that are exceedingly difficult to distinguish from those of the disease of artificial origin, runt disease, caused by the reaction of injected cellular homografts of lymphoid cells against the young and defenseless foreign hosts. On the other hand, diagnosis of runt disease in rats, at least in our experience, presents no difficulty (*see* Billingham and Silvers, 1959*a*). Its initial manifestation is a conspicuous exfoliation of the skin, originating around the urogenital and anal orifices and soon extending to the entire body surface, being particularly evident on the ears, tail, and abdomen (FIGURE 1). This abnormality is accompanied by thickening, loss of elasticity and tonicity of the skin, which acquires a pergameneous consistency. Because of this cutaneous rigidity the victims have considerable difficulty in flexing their limbs and adopt a rather characteristic posture. The ears are usually thick, rigid, and underdeveloped (FIGURE 1).

Histology of the skin. Histological studies have confirmed that a runt's skin is highly abnormal with respect to both its dermal and its epidermal components. The epidermis is very thick, with increased keratinization, parakeratosis, and acanthosis with an apparent squamous metaplasia of the sebaceous glands. There is an increase in the number of prickle cells and a pronounced irregularity of the normally plane dermoepidermal junction or interface (FIGURES 2 and 3). In extreme cases cleavage or separation of the epidermis from its underlying dermis occurs with bulla formation, and the formation of intraepidermal abscesses is by no means rare.

The cellularity of the dermis is frequently abnormally high, chiefly because of the presence of histiocytes, that is, large cells with poorly defined cytoplasmic borders and with faintly eosinophilic cytoplasm (FIGURE 3). Only rarely do lymphocytes contribute to this cellularity. With respect to its fibrous structure, there is apparently an increased number of reticular fibers at the expense of collagen.

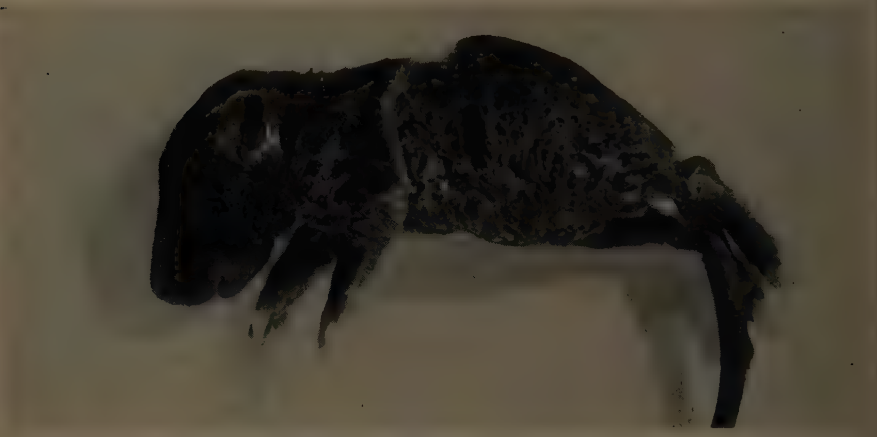

FIGURE 1. Fourteen-day-old B.N. rat suffering from acute runt disease as a consequence of its inoculation at birth with 6 million Lewis lymphoid cells. Note the conspicuous desquamation of the skin.

Although these abnormalities involve the integument in its entirety, they appear to be skin-specific in that they have not been observed in oral, pharyngeal, and respiratory epithelium or in the intestinal mucosa.

Diarrhea nearly always accompanies these symptoms; crusts and dried exudates usually cover the nares and eyes, and keratitis may occasionally be observed. The body temperature of affected individuals is usually at least 2° C. below that of normal, untreated littermates.

Differences in body weight between individuals inoculated with appropriate dosages of lymphoid cell-containing suspensions and untreated littermate controls usually become evident at 12 to 13 days postpartum, when the body weight and length of normal rats are increasing rapidly. As in affected mice, the development of rats is dramatically arrested by the disease, so that by about the seventeenth day their body weight is only about one half that of control subjects. Depending upon the type of cell inoculated, the dosage, and the donor/recipient strain combination, a variable proportion of the affected subjects eventually may recover completely, but recovery usually requires several weeks. Those that succumb usually die within 2 to 3 weeks.

The nodes and spleen. At autopsy the conspicuous abnormality of the runts is a dramatic enlargement of all lymph nodes and lymphoid tissues. Inguinal, axillary, cervical, and mesenteric nodes are enlarged by a factor ranging from 3 to 10. The spleen likewise is greatly enlarged, although here there is great weight variation from animal to animal in the initial stages of the disease. Although usually compact and of light red color, this organ sometimes presents a mottled surface. These abnormalities of lymph node and spleen weights are usually detectable at the sixth day, increasing in magnitude thereafter.

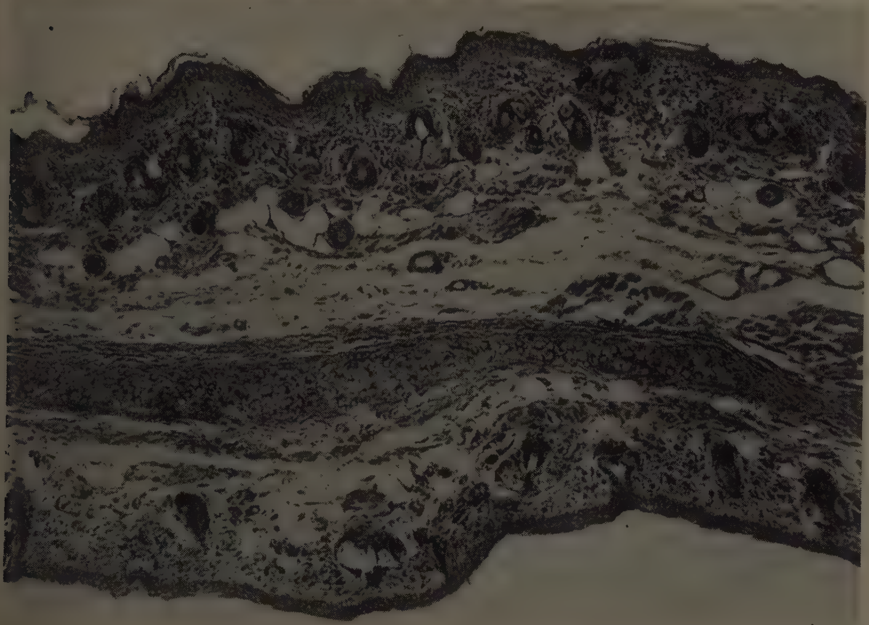

FIGURE 2. Section through ear of a normal 16-day-old Lewis rat. The superficial epidermis is 3 or 4 cell layers thick, with a very small amount of keratin. There is a considerable amount of collagen in the dermis. Hematoxylin and eosin stain. ×75.

In the enlarged nodes and spleens of these animals there is an almost total disappearance of organized lymphoid structures. Lymphoid follicles are not present, and only scattered lymphocytes and lymphoblasts are seen, with no particular distribution. The follicle areas or sites are replaced completely by reticulum cells and large, pale eosinophilic histiocytes; in the medullary sinusoids clumps of basophilic stem cells are frequently seen, and some of these foci seem to be engaged in erythropoiesis.

It is characteristic of runts that their thymuses, unlike their nodes, diminish in size, commencing by about the twelfth or thirteenth day. By the seventeenth day the entire organ may be represented by no more than a few small fibrous filaments. It is quite characteristic that the 2 parathymic lymph nodes undergo hypertrophy, so that from a cursory examination they may appear to replace the thymus.

The blood and hemopoietic tissue. In runts the cellular constitution of both the blood and the bone marrow undergoes a profound alteration from the normal picture. The more obvious changes are summarized briefly below.

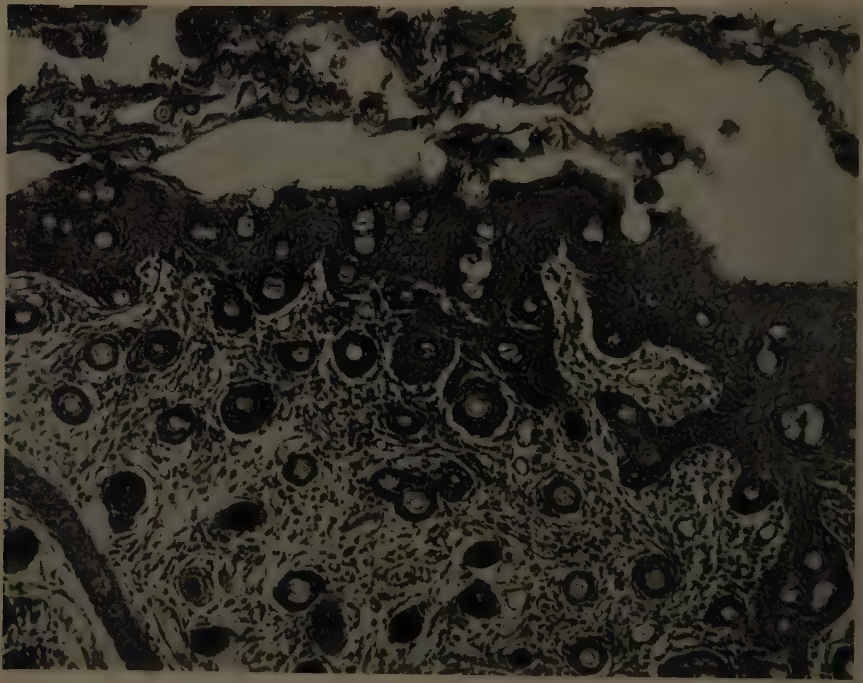

FIGURE 3. Section through ear of 16-day-old Lewis rat inoculated with 10 million B.N.-strain lymphoid cells at birth and suffering from acute runt disease. The epidermis is extremely thick, with a large amount of keratin, the dermoepidermal junction is very irregular, and the dermis shows increased cellularity, with histiocytes and fibroblasts. Hematoxylin and eosin stain. ×200.

In normal rats beginning at the fourth day of age the red cell count increases progressively from about 2.5 million/mm.^{3} to an average adult value of 7 to 8.5 million/mm.^{3}, whereas in the runts this progressive increase is not observed. Their red cell count remains steady at the 2.5 million/mm.^{3} level up to the terminal stage of the disease, representing a reduction of the normal by a factor of 3. The degree of anemia is actually higher than is suggested by these figures, since at the terminal stage their hematocrit values are slightly higher than in littermate controls, probably as a consequence of fluid loss through diarrhea.

Smears of peripheral blood show a high degree of anisocytosis and poikilocytosis with occasional spherocytes. Furthermore, a large proportion of the circulating red cells is polychromatophilic, with a preponderance of basophilic elements. Red cells with nuclear residues such as Jolly's bodies are occasionally present. Despite the high degree of basophilia, the amount of hemoglobin per red cell in the runt is comparable to that of appropriate controls. In accordance with a picture of anemia in the peripheral blood, the bone marrow prints reveal a high degree of hypoplasia of the erythrocytic series, with a shift of the granulocyte/erythrocyte ratio to approximately 4/1.

The changes in the white blood cells are even more striking from both qualitative and quantitative viewpoints. Commencing on the seventh or eighth day, the number of blood leukocytes rises very rapidly, usually reaching values of 30,000 to 40,000/mm.^{3} (control value, about 7500/mm.^{3}), although counts as high as 100,000/mm.^{3} have been recorded. This enormous increase is attributable chiefly to the granulocytes, which represent about 85 per cent of all white cells present in runts' blood. It may be emphasized that in the controls the lymphocyte is the predominant white blood cell type; usually 85 per cent lymphocytes and 15 per cent granulocytes are present. There appears to be no fall in the absolute number of lymphocytes.

Qualitatively, these granulocytes are profoundly altered. For example, the Arneth count shows a considerable deflection to the right, with 5 or 6 and even more nuclear lobations, while the cytoplasm of these cells is highly immature, with few granules and persistent basophilia. In the marrow, as already mentioned, there is a great predominance of cells of the myelogenous series, with a threefold or fourfold increase of eosinophils. In addition, numerous myelocytes with Rieder-like characteristics are seen.

The marrow and peripheral blood picture may best be described as a hypoplastic, dyschromic anemia with absolute granulocytosis and profound alteration of the maturation of granulocytes. Whether this highly abnormal picture is a direct expression of the immunological reaction of the grafted homologous "lymphoid" cells against their foreign host or is merely the secondary result of nutritional or metabolic alterations has yet to be determined.

Incidence of Runt Disease in Relation to Type and Dosage of Homologous Cells Inoculated

Once initiated, runt disease runs a constant clinicopathological course, irrespective of whether it was produced with homologous cells derived from the spleen, lymph nodes, or the lymphatic stream flowing along the thoracic duct. The finding that spleen cell suspensions are less harmful on a dosage basis than those prepared from nodes was to be expected from the results of similar studies on mice (Billingham and Silvers, unpublished data), and also from the fact that spleen contains relatively fewer lymphoid cells than nodes.

Comparison of the incidence of runt disease caused by the inoculation of lymph node cells (prepared from the pooled axillary, brachial, cervical, and mesenteric nodes) on the one hand, and by the inoculation of lymphocytes, obtained by cannulation of the thoracic duct on the other (Bollman, *et al.* 1948; Gowans, 1957), shows that these 2 types of cell suspension are almost indistinguishable with respect to their immunological competence when tested in the Lewis → B.N. combination. With the B.N. → Lewis combination the evidence is in fact suggestive that thoracic duct lymphocytes are more potent than node cells. It is remarkable that so few thoracic duct cells are required to cause runt disease; 2.5 million are effective for more than 50 per cent of the subjects, and even 1 million can kill the hosts. Even if due allowance is made for the fact that, in the mouse, lymph node cells are at least 3 times as effective in inducing tolerance as bone marrow cells on a strictly quantitative basis (Billingham and Silvers, unpublished data), the considerable disparity revealed in the present study between the number of bone marrow cells required to induce tolerance regularly and the much smaller number of node or thoracic duct lymphocytes that will cause runt disease suggests that the induction of tolerance by these cells is not a necessary prerequisite for the development of runt disease. This view is strengthened by our finding that, of the many animals that either failed to develop runt disease or that eventually recovered from it, none was found to be tolerant when test-grafted. The median survival times of adult skin homografts transplanted to the newborn recipients (*see* above, under *The fate of interstrain skin homografts*) indicate that even without the intercession of tolerance* the small numbers of lymphoid cells inoculated into newborn hosts have ample time to proliferate and to inflict immunological damage long before the latter have matured sufficiently to defend themselves. Indeed, immunologically procured debilitation is probably the major factor that guarantees the foreign cells what security of tenure they possess, especially with the B.N. → Lewis combination.

It is of some importance that the time of onset of the symptoms of runt disease and the time of death, if it occurs, appear to be almost completely independent of the actual number of homologous cells injected at birth over a wide dosage range (TABLE 2). This may be simply the outcome of the exponential rate of proliferation of the particular cell type responsible for the disease, at least during the early period following inoculation, combined with factors such as availability of sites for infiltration and colonization that restrict the ultimate size of the derived population.

The finding that lymphocytes obtained by cannulation of the thoracic duct are as competent immunologically as cell suspensions prepared from lymph nodes is highly important for the light that it sheds upon the identity of the cell type responsible for the immunological competence manifested by the leukocytes present in the whole blood of chickens and mice (Simonsen, 1957; Billingham, 1958; Cole *et al.*, 1959; and Terasaki *et al.*, 1959). Some member of the lymphocytic series was strongly suspected hitherto on the basis of the low dosage of leukocytes required to cause runt disease in the mouse and the preponderance of lymphocytes among murine white blood cells. Terasaki's (1959) work on chickens also implicates this type of cell very strongly.

In the rat the immunological competence of its thoracic duct lymphocytes provides a sufficient explanation for the activity of its blood leukocyte concentrates. The highly important question still to be answered is: What type of lymphocyte is responsible, since lymphocytes certainly do not constitute a homogeneous population? About 95 per cent of the cells in thoracic duct lymph are "small" lymphocytes that most authorities would probably dismiss outright as possible causal agents of runt disease on the grounds that they are

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* Despite the findings of Medawar and Woodruff (1958) in rats, with the present strains the grafting of skin to newborn animals confers no tolerance (Steinmuller, unpublished data).

"end" cells incapable of division, although their life span is probably considerable (Gowans, 1957; 1959). This leaves approximately 5 per cent so-called large and medium lymphocytes that are known to be capable of division (Gowans, 1957; Everett *et al.*, 1960) and cells of intermediate sizes for consideration. Preliminary attempts to make use of the known differential mortality of large and medium lymphocytes in cell culture to obtain pure preparations of small lymphocytes for investigation of their ability to cause runt disease have so far given inconclusive results, although in one experiment 3/3 rats succumbed to runt disease after they were inoculated with a preparation, cultured for 24 hours, that apparently was free from both large and medium lymphocytes. Unfortunately, attempts to attach significance to negative results obtained with preparations of this type are always open to the criticism that the survivors of the initial cell population, although viable, may have been harmed irreparably in a functional sense.

TABLE 2

INCIDENCE OF RUNT DISEASE FOLLOWING INTRAVENOUS INOCULATION OF NEONATAL RATS WITH VARIOUS TYPES OF TISSUE CELL SUSPENSION

Strain combination	Type of cell inoculated	Cell dosage (millions)	No. of animals	Incidence of runt disease (%)	Incidence of fatal runt disease (%)	Range of ages at death of runts (days)
Lewis → B.N.	Spleen	10–20	14	79	79	15–16
	Node cells	10–20	13	100	100	13–16
	Node cells	5	11	73	73	14–19
	Node cells	2.5	8	75	75	23–24
	Thoracic duct cells	30–40	14	100	100	12–17
	Thoracic duct cells	5	28	78	64	16–20
	Thoracic duct cells	2.5	30	67	67	15–27
	Thoracic duct cells	1	14	21	21	24–25
B.N. → Lewis	Spleen	30	9	44.5	11.0	16
	Spleen	10–20	31	0	0	0
	Node cells	20	29	34.5	34.5	16–19
	Node cells	10	26	42	15	16–21
	Node cells	5	24	54	25	16–21
	Thoracic duct cells	30–50	16	100	100	16–19
	Thoracic duct cells	20	22	86	73	16–23
	Thoracic duct cells	10	38	95	34	16–21
	Thoracic duct cells	5	35	86	11.5	16–18

An alternative approach, at present in progress, is based upon the fact that the actual proportion of large lymphocytes in thoracic duct lymph of Lewis rats varies within the limits 1 to 5 per cent. Hence, working with the Lewis → B.N. strain combination and injecting fairly low dosages (1 to 6 million thoracic duct cells), we are able to compare the incidence of runt disease first, with the total number of lymphocytes of all types inoculated, and second, with the actual number of large lymphocytes inoculated. Thus far the results obtained fail to indicate any relationship between the large lymphocyte content of an inoculum and its runt-inducing potentiality; on the other hand, there appears to be a clear-cut relationship between the absolute number of cells injected and the incidence of runt disease.

Discussion and Conclusions

The finding that cell dosage may be a highly important factor in the induction of tolerance of homologous cells, particularly with strain combinations in which the antigenic disparity is very great and failure to induce tolerance in newborn animals has resulted, should certainly be borne in mind before finally abandoning inoculation of neonatal subjects and going back to fetal inoculations with all the additional complications entailed. Furthermore, any meaningful experimental definition of the duration of the tolerance-responsive period must clearly take this dosage factor into consideration, especially in the case of older experimental subjects.

It is now well known that, under a variety of circumstances procurable by experimental or genetic artifice, "cellular" or even solid tissue homografts obtain some measure of tenure in hosts having antigens that are not shared in common with the inoculated cells. In each case a disease closely resembling runt disease may be the outcome, with subsequent death or recovery. These diseases have been variously named, according to the manner in which they have been produced or according to their more obvious symptoms, such as wasting disease, secondary radiation sickness, homologous disease, runt disease, runting syndrome, and parabiosis intoxication. These conditions have many clinicopathological features in common, (*see*, for example, Kaplan and Smithers, 1959; DeVries and Vos, 1959; Gorer and Boyse, 1959) and most authorities are now agreed, on the basis of an impressive body of experimental evidence, that they are all the consequence of a transplantation immunity reaction on the part of transplanted immunologically competent cells against "foreign" tissue transplantation antigens confronting them in their hosts (*see* Billingham, 1959). At present, however, it is probably fair to say that we are still like archeologists trying to determine from old relics on an ancient battleground exactly who the real contestants were, exactly how they fought, and what happened to them. One crucial issue is what happens to the immunologically competent cells that cause the damage. Are they destroyed as a consequence of their sensitization and contact with the cellular antigens concerned? The work of Boyce (1959) is of particular relevance here. Many of these problems have been very ably formulated and discussed in a recent paper by Kaplan and Rosston (1959).

References

Billingham, R. E. 1958. Studies on the reaction of injected homologous lymphoid tissue cells against the host. Ann. N. Y. Acad. Sci. **73**(3): 782.

Billingham, R. E. 1959. Reactions of grafts against their hosts. Science. **130:** 947.

Billingham, R. E. & L. Brent 1959. Quantitative studies on tissue transplantation immunity. IV. Induction of tolerance in newborn mice and studies on the phenomenon of runt disease. Phil. Trans. Roy. Soc. London. **B242:** 439.

Billingham, R. E., L. Brent & P. B. Medawar. 1956. Quantitative studies on tissue transplantation immunity. III. Actively acquired tolerance. Phil. Trans. Roy. Soc. London. **B239:** 357.

Billingham, R. E., L. Brent & N. A. Mitchison. 1957. The route of immunization in transplantation immunity. Brit. J. Exptl. Pathol. **38:** 467.

Billingham, R. E. & W. K. Silvers. 1959a. The induction of tolerance of skin homografts in rats with pooled cells from multiple donors. J. Immunol. **83:** 667.

Billingham, R. E. & W. K. Silvers. 1959b. Inbred animals and tissue transplantation immunity. Transplantation Bull. **6:** 399.

Bollman, J. L., J. C. Cain & J. H. Grindlay. 1948. Techniques for the collection of lymph from the liver, small intestine, or thoracic duct of the rat. J. Lab. Clin. Med. **33:** 1349.

Boyse, E. A. 1959. The fate of mouse spleen cells transplanted into homologous and F_1 hybrid hosts. Immunology. **2:** 170.

Cole, L. J., R. M. Garver & J. P. O'Kunewick. 1959. Lethal graft-versus-host reaction induced in X-irradiated F_1 hybrids by parental strain leucocytes. Transplantation Bull. **6:** 429.

De Vries, M. J. & O. Vos. 1959. Delayed mortality of radiation chimeras; a pathological and hematological study. J. Natl. Cancer Inst. **23:** 1403.

Egdahl, R. H., F. R. Roller, R. L. Swanson & R. L. Varco. 1958. Acquired tolerance to homografts and heterografts in the rat. Ann. N. Y. Acad. Sci. **73**(3): 842.

Everett, N. B., W. O. Reinhardt & J. M. Yoffey. 1960. The appearance of labeled cells in the thoracic duct lymph of the guinea pig after the administration of tritiated thymidine. Blood. **15:** 82.

Gorer, P. A. & E. A. Boyse. 1959. Pathological changes in F_1 hybrid mice following transplantation of spleen cells from donors of the parental strains. Immunology. **2:** 182.

Gowans, J. L. 1957. The effect of the continuous re-infusion of lymph and lymphocytes on the output of lymphocytes from the thoracic duct of unanaesthetized rats. Brit. J. Exptl. Pathol. **38:** 67.

Gowans, J. L. 1959. The recirculation of lymphocytes from blood to lymph in the rat. J. Physiol. **146:** 54.

Kaplan, H. S. & B. H. Rosston. 1959. Studies on a wasting disease induced in F_1 hybrid mice injected with parental strain lymphoid cells. Stanford Med. Bull. **17:** 77.

Kaplan, H. S. & D. W. Smithers. 1959. Auto-immunity in man and homologous disease in mice in relation to the malignant lymphomas. Lancet. **2:** 1.

Lengerová, A. 1957. Effect of irradiation during embryogenesis on the relationship between the maternal organism and offspring from the aspect of tissue compatibility. Folia Biol. **3:** 333.

Medawar, P. B. 1957. The immunology of transplantation. Harvey Lectures. 1956–1957. **53:** 144.

Medawar, P. B. & M. F. A. Woodruff. 1958. The induction of tolerance by skin homografts on newborn rats. Immunology. **1:** 27.

Simonsen, M. 1957. The impact on the developing embryo and newborn animal of adult homologous cells. Acta Pathol. Microbiol. Scand. **40:** 480.

Terasaki, P. I. 1959. Identification of the type of blood-cell responsible for the graft-versus-host reaction in chicks. J. Embryo.. Exptl. Morphol. **7:** 394.

Terasaki, P. I., J. A. Cannon & W. P. Longmire. 1959. Mortality of chick embryos upon injection of homologous adult cells. Proc. Soc. Exptl. Biol. Med. **100:** 639.

Woodruff, M. F. A. & L. O. Simpson. 1955. Induction of tolerance to skin homografts in rats by injection of cells from the prospective donor soon after birth. Brit. J. Exptl. Pathol. **36:** 494.

Woodruff, M. F. A. & M. Sparrow. 1957. Further observations on the induction of tolerance of skin homografts in rats. Transplantation Bull. **4:** 157.

Discussion of the Papers

Morten Simonsen: Certainly, I shall not present any new data.

In Siskind's paper I think there are two things of very great importance. The first is his demonstration of three different methods of preventing the runting disease. There is nothing there in either his methodology or in his conclusions with which I disagree.

What challenges me, on the other hand, is that part of his paper in which he describes a serial transfer of runt disease because this, as you probably will have noticed, is in direct contrast to the results I have reported.

I do not know the explanation of the difference. One difference in the experimental setup, of course, is that I grafted from adult parental strains to F_1 hybrids, whereas Siskind did his grafting to newborn homozygous mice.

The unfortunate thing about it is that, from theoretical expectations, one would predict from these two different experimental arrangements conclusions exactly opposite from those that the actual results have given. This may be another example of what I think is almost a general acceptance on the part of contributors to this monograph that so many of the theoretical predictions that appeared sound a few years ago now seem, to some extent, open to question.

It seems to be very difficult to generalize from one antigenic situation to the other, and the only thing I suggest is that Siskind try to repeat his experiment with F_1 hybrid hosts. I shall repeat mine with homozygous hosts.

I ask further if Siskind has carried the serial transfer further than to a second host. I wonder whether, on repeating it for ten passages, he would still be able to produce runt disease or whether he would end up with something like the exhaustive sensitization that I have described.

As for Billingham's paper: I think it is most important that he has shown so clearly to us how important the dose and time relations are for any statement of the length of the inactive or the adaptive period. One thing that interested me very much in his presentation was the dermatitis he obtained in the runting rats.

That is quite unknown in so far as I know from mice, and I ask if he has tried to elucidate the pathogenesis of this dermatitis by seeing whether it would extend to homografts from the same strain used for production of the runt disease. One would hesitate to predict on theoretical grounds what the result would be, but I should like to know the facts.

J. R. Battisto (*Albert Einstein College of Medicine of Yeshiva University, New York, N. Y.*): In observing runted animals and animals that undergo secondary disease, one is struck with the thought that each recipient is, by virtue of being immature or X-irradiated, under an immunological handicap. I direct your attention, then, to a syndrome resembling secondary disease or runt disease that can be caused to occur in normal nonirradiated adult guinea pigs weighing 400 to 900 gm. that, apparently, are not immunologically handicapped.

The condition has been termed "erythematous disease" because adult normal recipients of lymph node or spleen cells taken from certain normal guinea pig donors develop a characteristic erythema over their entire bodies. In addition, the recipients exhibit spikes of intermittent fever, express pain on handling, and show progressive weight loss. About one half of the stricken animals recover; the remainder die within five to forty days after transfer.

The original observation of this syndrome was made several years ago in collaboration with Chase when we were engaged in other unrelated experiments. Since then, I have been able to show that erythematous disease can be induced, using normal donors, provided these donors show a naturally occurring delayed cutaneous reaction to homologous serum. Twenty per cent of the guinea pigs of the closed stock from The Rockefeller Institute, New York, N. Y., have this property. Donors are also characterized by a lack of the serum factor found to exist in other guinea pigs and, when dermally inoculated, it evokes the delayed skin reaction.

Erythematous disease can be induced when the donor to recipient ratio is as small as 1:1.

I ask Billingham whether he has seen any characteristics of runt disease, other than runting, in adult recipient animals given large numbers of donor cells.

SILVERS: I ask Siskind to make one comment on some results that have puzzled me. If I have understood him correctly, he is able to protect against runt disease by injecting homologous and isologous spleen cells on the same day, but one day later it is too late to protect with isologous cells, and injected animals die of runt disease. On the other hand, he is able to protect against this disease by introducing antiserum as late as two days after birth. In other words, the antiserum is apparently much more efficient than the isologous immunologically competent cells. From what we know about the homograft reaction, is this not surprising? Are there reasons that might explain it?

MCBRIDE (*McGill University, Montreal, P. Q., Canada*): May I comment on the problem of dosage? Working with adult rabbits, and using epithelial cell injections, we have been very unsuccessful in achieving immunity when we inject large numbers of cells. When we descend into the range of less than 50,000 epithelial cells per injection, using albinos, Dutch rabbits, and grays, we get something that approximates the second-set reaction. The work in progress at McGill now, therefore, is an attempt to ascertain the gradation responses according to dosage because it seems to us that we are encountering a regular phenomenon, where a low dosage of cells will give immunity and where the high dosage gives tolerance, which is the same thing that occurs with chemical antigens.

KOPROWSKI: Possibly Siskind mentioned it, but I nevertheless have a question about the "cured runts." Has their tolerance been checked? Furthermore, if they are tolerant, how long did this tolerance persist after they were "cured?"

SISKIND: First, with regard to the apparent inconsistency between Simonsen's experiments and ours: I feel that this can be accounted for, although not necessarily explained with certainty, by differences between the experimental designs employed.

In the first place, we have been using different strain combinations of donors and recipients. In the second place, Simonsen used 10- and 19-day-old donors, whereas we used 8-week-old A/Jax donors for the serial transfer of the runting syndrome. We used runts that had been alive, or had survived, for eight weeks before serial transfer as spleen cell donors in the transfer experiments.

I think that these differences are probably sufficient to account at least for the fact that we have differences in results. In so far as an explanation for these differences is concerned, I do not think we have one. I do think that there is at least one possibility that must be considered. In Simonsen's strain combination, a more mild form of the runt disease was observed than in our combination. Our animals die in 50 to 60 per cent of the cases. I think Simonsen's die in only about 10 per cent, if he allows them to go on.

This more mild runt disease might well reflect a less marked immunological disparity between Simonsen's donors and recipients than between our donors and recipients. The close immunological relationship that might possibly exist would probably favor the establishment of a state of immunological unresponsiveness, such as exists, according to Simonsen, in his animals; in our animals, which are immunologically more distantly related, shall we say, we do not obtain this state of immunological unresponsiveness as readily.

I think that one must keep in mind at all times in this sort of work that an animal that is called a runt and looks like a runt, when it matures can be a very different animal. To look at it and to say it is runted and small in no way gives one the right to make very many conclusions about its immunological or cytological status. Moreover, the way it looks at one particular moment in time does not necessarily reflect what is going on at that moment, but rather what has happened to it in the past. This is also a problem that must be faced, one that, at the moment, we cannot face. It would probably be of importance in explaining the differences in our results.

With regard to further serial transfers: we have done such transfers only in the C57 Black, DBA/2 passage, and then only once more past the first transfer; in other words, only to the second-set transfer. In this case, we sacrificed all of our donors. We also sacrificed all of the recipients and looked only for splenomegaly and liver lesions; a few of these animals did show the chronic liver lesions that are so commonly seen in runted mice. However, such a lesion may not necessarily be a specific one. Therefore, I fear that we have no answer to what does happen.

In regard to skin lesions: generally, we do not see skin lesions in the mouse, and therefore there is no further comment in this connection.

One thing of interest in the results reported in Russell's paper, especially since they agree with ours, is the fact that the gross criterion that we use for runt disease and for death probably makes our system a little less sensitive and probably explains why we observe no protection a day later, whereas Russell probably uses a more sensitive indicator, that of failure of weight gain, and he may observe protection a day later.

With regard to our ability to protect with serum and to Russell's inability to protect with his serum, I can suggest only the possibility that we are using a different strain combination. We are using C57 suspensions in DBA/2 hosts; he is placing DBA/1 cells into C57. I think that it might be of interest to attempt to reverse the situations with each of our systems.

As regards Silvers' question as to the efficacy of serum two days after birth and the failure of cells one day after birth: I think I can explain this, unlike many things that I cannot explain. When we give cells, we give a unit that is competent to do something, but is not competent to do it at that instant in time. The cells must be sensitized, and sensitization of cells requires a certain latent period. This latent period is not thoroughly known, but it probably is at least 24 to 48 hours. I think that the earliest that one has seen evidence of graft rejection is at 48 hours, in goldfish scales. Therefore I feel that when one gives serum two days after birth, as distinguished from giving cells one day after birth, the serum circulates much more rapidly, I think, than do our cells, which we give intraperitoneally. Second, I think that we are giving an agent that is effective at the instant of administration, rather than an agent that takes some time to become effective. Moreover, I think that if we accept this as an explanation, then a prediction from this is that immunized cells given one day later would protect, as distinguished from the action of normal cells given one day later. I thank Russell for providing in his paper the data and evidence to support this concept.

BILLINGHAM: When Simonsen asks the question, "Have you done so-and-so?" one is always very interested in the suggestion; and, if one has not already done it, one immediately undertakes it. Fortunately, this need not be regarded as merely a suggestion; it may be interpreted as the blessing of an enterprising being.

Both Simonsen and Battisto have asked if I have ever seen anything resembling a cutaneous manifestation in runt disease in adult animals. The answer, I fear, is very simple: unfortunately, no! However, I have never had any occasion to look for it.

Nevertheless, there is a situation well documented in the literature that appears in Finerty's review on parabiosis in *Physiological Reviews* a few years ago. In this article Finerty discusses parabiotic intoxication after parabiosing animals in which he says many workers have noted a reddening of the ears and feet. Our pathologist, V. Defendi, looking at the animals we had injected one day, said, "Have you looked at their ears and feet?" and I think Silvers and I said "No," and promptly did so. To our surprise the ears were flabby and erythematous, and so were the feet.

In other words, I think there is, in this condition of the ears and feet, and in the observations of parabiosis workers, something that Battisto has in mind and something that makes me still feel confident that my general argument and inclusion of parabiotic intoxication in types of graft-versus-host reaction really have not taken a heavy defeat, after all, in Eichwald's rebuttal earlier in these pages.

ERYTHROCYTES AS A TOOL IN STUDIES ON RABBIT RADIATION CHIMERAS AND SECONDARY DISEASE*

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The infusion of bone marrow into animals exposed to lethal irradiation has been shown to effect a considerable reduction in early mortality, as a result of repopulation of the depleted marrow by hematopoietic tissue.[1] When homologous or heterologous marrow is used, a wave of deaths frequently follows acceptance of the graft. Many of these delayed deaths are ascribed to a syndrome variously called radiation sickness, homologous disease, or wasting disease.[2] A previous report[3] from our laboratory dealt with the occurrence of delayed deaths in irradiated rabbits receiving infusions of homologous bone marrow, and it was suggested that some of these deaths were the result of a graft-versus-host reaction. Some features indicative of an immune hemolysis were noted.

The present study was undertaken to confirm and evaluate the occurrence of hemolysis in rabbit radiation chimeras and to establish whether it was the result of an immune response on the part of graft or host cells. In addition, a comparison was made between the mortality figures resulting when marrow was injected immediately (within 3 hours) and when injected 72 hours after irradiation.

Methods

Male albino New Zealand rabbits of about 2.5 kg. were used as recipients and young females of the same strain as donors. The animals were not, in the genetic sense, in any way inbred. The donor-recipient combinations were paired so that they differed in at least one antigen on the basis of Cohen's Hg^{ADF} allelomorphic system.[4] Recipients were exposed to 1100 r, as a split dose of 600 r followed 24 hours later by 500 r, and were injected intravenously, either immediately (group E) or 72 hours later (group D) with 0.6×10^9 to 1.5×10^9 nucleated bone marrow cells.[5] One control group (A) received irradiation without support; a second group (B) received twice weekly an intramuscular injection of 100,000 U. of penicillin and 100 mg. of streptomycin; a third group (C) received marrow, which had been treated before infusion by sonication, followed by lyophilization and storage at −20° C. for several weeks. Groups C, D, and E all received the same antibiotic regimen as group B.

Mortality

The mortality figures at 15 days and at 8 and 12 weeks are summarized in TABLE 1. The animals that received no treatment other than antibiotics (group B) had a 15-day mortality of 72 per cent compared to 91 per cent for the con-

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trol group A. No deaths in group B were observed after 15 days. Additional protection was not obtained by the use of treated marrow (group C). When fresh marrow was given immediately (group E) or 72 hours after irradiation (group C), the 15-day mortality was considerably reduced, but in contrast to the other groups (A, B, C) in which death occurred only rarely after this time, there was a spate of deaths between the third and eighth weeks. The result was a higher 8-week mortality in group D than in the control groups B and C. In group E, however, in spite of delayed deaths, the 8-week mortality was 57 per cent as opposed to 70 per cent in group C.

Transplant Results

The success of the transplant was judged by the presence of donor erythrocytes or leukocytes in the circulation of the recipient. The former were determined weekly by the Dacie and Molison modification of the Ashby differential agglutination technique,[6] and the latter, twice weekly for the first month and thereafter once weekly, by the presence of female leukocytes in Wright-stained blood smears.[7] As measured by these criteria, no successful transplants were obtained when sonicated lyophilized stored marrow was used (group C). On the other hand, when fresh marrow was used, a successful transplant was obtained in all but one instance. However, complete "takes" (that is, the presence of both erythrocytes and leukocytes of the donor) occurred in only 18 of 33 chimeras surviving at 15 days. Eight had only donor erythrocytes and 7 had only donor leukocytes. Furthermore, of 18 animals surviving at the end of 12 weeks donor cells had disappeared from 6, so that only 12 rabbits had a successful 12-week graft. In 8 of these the graft was complete in that the chimera had both erythrocytes and leukocytes (TABLE 2).

TABLE 1
MORTALITY FIGURES

Group	No. of animals	Mortality (%)		
		15 days	8 weeks	12 weeks
Control animals				
Group A (no treatment)	33	91	94	94
Group B (antibiotics)	50	72	72	72
Group C (antibiotics + treated marrow)	13	70	70	70
Transplanted animals				
Group D (antibiotics + fresh marrow 72 hours postirradiation)	35	56	83	83
Group E (antibiotics + fresh marrow immediately after irradiation)	28	39	57	57

Immunological Studies

In our laboratory the normal disappearance slope of Cr^{51}-labeled cells gives an apparent half life of 18 ± 2 days. Experiments had shown that the normal disappearance slope is not observed when cells are infused immediately after irradiation. Instead, when counts are plotted against time on a semilogarithmic scale, a plateau is obtained for approximately 7 days after X-rays, followed by a sharp but variable drop in radioactivity as a result of hemorrhages.[8] Preliminary experiments in which erythrocytes were injected into irradiated and transplanted animals showed similar results. Consequently, Cr^{51}-labeled cells were infused into the radiation chimeras 3 to 4 weeks after irradiation and transplantation. Erythrocytes were taken from the donor or the recipient before the start of the experiment and stored in the frozen state in glycerol at −20° C. When needed, the cells were thawed, the glycerol was removed, and the cells were labeled with Cr^{51}. We have shown that cells stored in such a manner for at least 7 weeks have a normal *in vivo* survival time and retain their antigenicity (unpublished observations).

Donor cells were infused into 12 chimeras, and a normal survival time was observed in all but 1 animal in which the cells had a shorter than normal half life. When, however, recipient cells were infused into 8 other chimeras, a greatly shortened half life of 8 to 12 days, commensurate with a hemolytic process, was observed. A normal half life of recipient cells was noted in one animal, which was later shown not to have accepted its graft.

TABLE 2
RESULTS OF MARROW TRANSPLANTS

Fate of graft	Nature of graft			
	Complete (RBC and WBC)	Incomplete		Total
		RBC	WBC	
Permanent graft*	8	1	3	12
Graft reversal†	3	1	1	5
Delayed death‡	7	5	3	15
No take				1

* Graft present at 12 weeks.
† Graft present at 2 weeks, but not at 12 weeks.
‡ Animals dying between second and eighth weeks while still carrying graft.

Further evidence of an immune response was the presence, at both 3 and 7 weeks, in the sera of 26 chimeras of antibodies to stored host erythrocytes. These were detected by a positive result of an indirect Coombs' test. In only 3 cases of 20 was the indirect Coombs' test result also positive with stored donor erythrocytes (one of these 3 animals showed a shortened half life of donor cells). These 3 animals died before the eighth week. The Coombs' test gave a negative result with both donor and host cells in the 1 animal that at no time showed evidence of a successful transplant.

Discussion

These results indicate that immunologically competent cells are transfused with bone marrow and form antibodies to host antigens. When one of these antigens is erythrocytes, the antibodies formed cause an immunologically specific hemolytic anemia that is probably an indicator of a more generalized reaction of the graft against the host. This immunological reaction on the part of the graft is not necessarily lethal, since some rabbits do survive, but it appears to occur to a greater or lesser extent in all the animals. With the dose of irradiation used in this study (1100 r), permanent suppression of the host's immune mechanism against the graft does not always result for, although initially there were almost 100 per cent successful takes, of the 18 animals that survived at 12 weeks only 12 retained their grafts, as judged from the presence of donor erythrocytes or leukocytes. Furthermore, irradiation at 1100 r does not always permit a complete transplant take, as many chimeras have donor leukocytes or erythrocytes, but not both. Similar results have been observed by Welling *et al.*[9] in irradiated mice transplanted with rat bone marrow.

This situation is at its worst when recovery of the host's immune mechanism occurs in the presence of a graft that is actively producing antibodies against the host. Such a situation in our animals is incompatible with life.

Acknowledgments

We express our thanks to James Dealy, Jr. and to the Department of Radiology, Peter Bent Brigham Hospital, for their collaboration on the irradiation of the animals. Mary Bradford, Colette Hanau, Caryl Magnus, and Joan Voorhees all provided excellent technical assistance.

References

1. Ford, C. E., J. L. Hamerton, D. W. H., Barnes & J. F. Loutit. 1956. Cytological identification of radiation-chimeras. Nature. **177:** 452–454.
2. Trentin, J. J. 1958. Tolerance and homologous disease in irradiated mice protected with homologous bone marrow. Ann. N. Y. Acad. Sci. **73**(3): 799–810.
3. Lee, F., J. B. Dealy, Jr., G. J. Dammin & M. S. Brooke. 1958. The homograft reaction in X-irradiated rabbits treated with homologous bone marrow: a preliminary report. Ann. N. Y. Acad. Sci. **73**(3): 825–833.
4. Cohen, C. 1958. On blood groups and confusion in the rabbit. Transplantation Bull. **5:** 21–23.
5. Porter, K. A. & J. E. Murray. 1958. Long term study of X-irradiated rabbits with bone marrow homotransplants. J. Natl. Cancer Inst. **20:** 189–205.
6. Dacie, J. V. & P. L. Mollison. 1943. Survival of normal erythrocytes after transfusion to patients with familial haemolytic anemia. Lancet. **1:** 550–552.
7. Porter, K. A. 1957. Use of sex differences in morphology of polymorphonuclear leucocytes to indicate survival of marrow homotransplants. Transplantation Bull. **4:** 129.
8. Piomelli, S. & M. S. Brooke. Hematological studies of X-irradiated rabbits. Acta Haematol. In press.
9. Welling, W., O. Vos, W. W. H. Weyzen & D. W. van Bekkum. 1959. Identification and follow-up of homologous and heterologous bone-marrow transplants in radiation-chimeras. Intern. J. Radiol. Biol. **2:** 143–152.

Part V. Organ Transplantation

TRANSPLANTATION OF HOMOLOGOUS PARATHYROID GLAND IN THE TREATMENT OF CHRONIC TETANY: THREE- TO SEVEN-YEAR FOLLOW-UP ON THREE PATIENTS

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Three patients have been observed since November 1952 in whom homologous transplantation of thyroid and parathyroid glands has been accomplished. In these 3 patients clinical and laboratory observations had been reported as of 1½ to 5 years since transplantation.[1] The purpose of this communication is to report observations over an additional 2 years.

The procedure followed in each case was that of anastomoses among the vascular pedicles of the gland (carotid arteries and jugular veins) with available vessels in the patient.

In Case 1 the anastomoses were accomplished in the left inguinal canal. In Case 2 the anastomoses were accomplished to the epigastric vessels under the right rectus muscle. In Case 3 the anastomoses were accomplished in the right saphenofemoral triangle.

At this time, 3½, 4½, and 7 years following homologous transplantation, the patients are generally free from symptoms of thyroid and parathyroid deficiency. Medications for both thyroid and parathyroid deficiencies had been administered prior to transplantation.

Clinical, endocrinological, chemical, and radiological examinations have been done at intervals.

Donor tissue was obtained as follows:

Case 1. A premature infant who had died 5 days after delivery because of prematurity. This blood type was not identified (the patient for donor No. 1 was blood type AB).

Case 2. Donor gland was obtained from a 5-month-old infant who died following sudden decompression of a myelomeningocele. Donor blood type was O, Rh positive (cDe). The patient was blood type O, Rh positive.

Case 3. The donor gland was obtained from an infant 1 month of age who died of pneumonia. The donor blood type was O, Rh negative (cDe). The patient was blood type B, Rh positive.

In every case the donor gland was obtained after the proper arrangements were made, starting 1 hour following the infants' death.

Case Reports

Case 1. Patient E.M. (B.) was 27 years of age at the time of transplantation on November 6, 1952.

Tetany had existed for 11 years, between 1941 and 1952. In 1948 the patient's serum calcium was 5.7 mg. per cent, and serum phosphorous was 6.5 mg. per cent. Her therapy now (1959) consists of a teaspoon of calcium once or twice a day during menstrual periods. Examination reveals her weight to be 104 lb. (it was 135 prior to transplantation in 1952). Physical examination is normal, except for incisional scars in the neck, abdomen, and groin. There is a palpable mass in the left inguinal canal at the site of the transplantation, which is approximately 3 cm. in diameter.

Chemical examinations on February 7, 1959 revealed a serum calcium of 8.8 mg. per cent serum phosphorus of 4.2 mg. per cent, and blood urea nitrogen of 22 mg. per cent. Blood count was normal except for a slight anemia (10.7 gm. hemoglobin), and urinalysis revealed a few white cells, a few red blood cells, and occasional granular casts.

Blood pressure has been maintained at 100 to 105 over 75 to 80. The menstruation cycle is from 22 to 25 days, with a 3- to 6-day flow.

This patient was married on September 20, 1958. She had no menstrual periods between December 1958 and March 1959. There was a positive pregnancy test in February 1959, but she aborted in March 1959. There was also a period of amenorrhea between August 1959 and October 1959, again associated with positive signs of pregnancy. However, she again aborted during the first week in October.

This patient's last menstrual period was in November 1959. A pregnancy test was positive on January 18, 1960 (she aborted again in February 1960).

Case 2. Patient M.A. (C.) was 35 years of age at the time transplantation was done in 1955.

Hypoparathyroid tetany had been present for 15 years. In March 1959, because of weight gain and sluggishness, this patient took 0.5 mg. thyroxine and about 3 teaspoons of calcium gluconate daily. However, she could not accept these dosages without developing extreme agitation, weight loss, and signs of hyperthyroidism. From May 1959 to the present she takes only 0.25 mg. of thyroxine twice weekly and 1 teaspoonful of calcium daily to be asymptomatic.

Laboratory studies in August 1959 by John A. Crago of Gainesville, Fla. revealed the calcium to be 9.1 mg. per cent, the phosphorus 3.9 mg. per cent, and the serum cholesterol 322 mg. per cent. Blood count and urinalysis were normal, serum proteins were normal, and the patient was cheerful, happy, and working every day. She did not desire to become pregnant.

Case 3. Patient M. (G.) was 36 years of age at the time of transplantation in April 1956.

Tetany had been present for 14 years. This patient was able to remain free from tetany, although she showed elements of hypothyroidism for the first 1½ years after transplantation. However, during the second year following transplantation, evidence was obtained that thyroid function was improving. Furthermore, during hospitalization in August 1959 a significant pick-up of radioiodine was identified at the subinguinal area site of transplantation.

In August 1959 serum calcium was 8.6 mg. per cent, serum phosphorus was 4.2 mg. per cent, and blood urea nitrogen was 18 mg. per cent. Urinalysis and blood count were normal. Serum proteins were 6.3 gm. per cent, with the normal albumin-to-globulin ratio. This patient is suffering from arthritis, bursitis, and recurrent episodes of cystopyelitis. She is taking 1 or 2 teaspoons of calcium daily during menstruation.

Summary

Three patients who had hypothyroid and hypoparathyroid states beyond adequate medical control had homologous transplantation of thyroid and parathyroid glands, using vascular anastomoses among the jugular and carotid pedicles to available arteries and veins in the hosts.

Examinations 3½, 4½, and 7 years after each respective transplantation indicate a maintained asymptomatic state insofar as parathyroid function is concerned. In 2 cases thyroid gland deficiency is absent, as demonstrated by radioiodine pick-up.

Reference

1. Sterling, J. A. 1958. Transplantation of homologous thyroid and parathyroid glands. Transplantation Bull. **5**: 50–52.

Discussion of the Paper

Sterling (*interviewing Case 1 patient, Erma B.*): Are you taking any medication for thyroid deficiency?

Patient: No.

Sterling: Are you taking any medications for a parathyroid condition?

Patient: No.

Sterling: Are you taking any calcium? Let's put it that way.

Patient: Well, small amounts.

Sterling: When do you take it?

Patient: Occasionally, a teaspoonful a day.

Herbert Conway (*Cornell University Medical College, New York, N. Y.*): At the outset, may I say that although seeing is said to be believing, I do not share Sterling's enthusiasm, although I think that he is to be applauded for his persistent efforts. It was from his work and that of Jordan that I became interested in this field three or four years ago.

I hesitate to be skeptical, but William Nickel and I have gleaned from the literature all the information we have to date on this condition regarding the transplantation of pre-adapted portions of the parathyroid tissue, and also the cases in which the tissue has been transplanted by vascular anastomosis.

In this historical review, one sees failure, failure, and more failure, until one reaches the case reported by Sterling and Goldsmith; and we have heard a report on 2 other cases today by Sterling.

These cases are listed as successes, but one may see in the postoperative medication, for example, that Case 1, Erma B. takes a teaspoon of calcium during menstrual periods, and you heard Erma B's report today that she takes a teaspoonful of calcium a day. Therefore, I think other observers might just as easily list such a case as a failure. I hope that Sterling will clarify this point, because it is terribly important. This business of calcium metabolism, it need not be stated, is not simply the measurement of the blood calcium in milligrams per cent at any one period of time, especially since this is only a transient measurement. A patient who is put in bed can perhaps mobilize calcium from her bones into her blood stream and feel very well after such an operation. Actually, to determine even slightly the postoperative state of Ca metabolism, total calcium balance studies are required.

Rowan Nicks is a very intrepid Australian who has transplanted two thyroid glands to the groin. He has also transplanted spleens by a vascular anastomosis, but he has explored these cases postoperatively and found nothing but fibrous tissue in the area of transplantation. I learned of this in direct communications from him.

Our patient, a case described by Nickel, Smith, and myself proved to be a failure after five months. It was very thrilling to see the gland *in situ* at the operating table, to see it pulsate immediately after it had been removed from its little cadaver "premature" or "premie" donor only five hours previously. We too enjoyed, in our case, Sterling's enthusiasm.

One might also cite the case of Watkins and his colleagues, which has been listed as a success, but I understand that Murray has it well documented and that now it is no longer looked upon as a success.

Therefore I believe that, in order to establish accurate or even acceptable criteria for complete function of a parathyroid gland homograft, one must be able to produce calcium balance studies demonstrating, first, a deficiency in calcium before homografting; second, satisfactory equilibrium of metabolism of calcium with the absence of oral or parenteral replacement therapy after the homografting; and, third, a very desirable but not practicable test in the human would be the histologic evidence of functioning parathyroid tissue; and, fourth, if possible, fulfillment of what might be called the Koch's postulate of endocrine transplantation, namely the return of the preoperative deficiency state after removal of the graft which, I state again, unfortunately is unpracticable in humans.

This is an intriguing field of endeavor, one which unquestionably should be pursued, and one for which Sterling, as well as others active in this field, deserve great credit. Still, there is no information as to whether these transplants in vascular anastomosis are rejected by the homograft rejection phenomenon, or whether, for other reasons, they fail because of a lack of perfection in technique. Undoubtedly, the solution of these problems will be rewarded by the development of successful transplantation mechanisms for the reversal of a great number of pathological disorders.

Richard E. Wilson (*Peter Brent Brigham Hospital, Boston, Mass.*): There is one important phase that Conway did not touch upon: the aspect of adaptation. This has been mentioned in the literature by many, especially by Jordan. We have studied this problem in both animals and in humans.

We used dogs, attempting to perform homografting of parathyroid tissue within Millipore chambers. The great majority of animals, after being supported through the initial total parathyroidectomy, were able to raise their serum calcium up to a normal level.

We were then quite disappointed to find that, when we removed the chambers after an initial period of varying length, more than 60 per cent of our animals were able to maintain a normal level of serum calcium for many months, in fact, for more than a year.

The other 40 per cent of the animals required more medication to stabilize their calcium and never were able to achieve a normal level of serum calcium but, after removal of their chambers, they too continued to do well clinically. They never had tetany. They showed no difficulties with weight gain, but maintained a serum calcium level between 6 and 8 mg. per cent, whereas the other 60 per cent of the animals maintained a calcium level of between 9 and 11 mg. per cent. All of these animals had been in tetany immediately after total parathyroidectomy.

We felt that this was an adaptive situation in some way, and we went on to study these animals further in an attempt to identify their response to stress and to find out in what way they were different from normal animals because, although they had normal serum calciums, we knew that they had no parathyroid tissue. It is interesting that we had a very significant statistical study where we gave these animals infusions of either calcium or EDTA, a *chelating* agent. It made no difference whether the parathyroidectomized dogs had a normal serum calcium or a low serum calcium. They all responded the same way when we calculated as per cent, the preinfusion value of serum calcium.

When these animals were given an EDTA infusion, they had a steep fall in serum calcium, and it took them many days in most cases and, certainly, many more hours to return to their level of preinfusion, as compared to the normal. Furthermore, when you gave them a calcium infusion, they raised their level of serum calcium much higher than the normal dogs did, and it took them many hours or days to return to the normal level.

Accordingly, what we see here is an animal that simulates the normal in many respects, but that actually is a much more inefficient animal with regard to calcium metabolism. It is entirely possible since, after all, hormones serve primarily to catalyze reactions that are already occurring in the body; it is the efficiency of this calcium metabolism, then, for which the hormone is responsible.

The patient referred to by Conway, as reported by Watkins, is the man whom we studied about 2½ years after a vascularized graft had been in place. For over a year he had required no medication. He had been thought to be perfectly well, and his last serum calcium was normal. However, when we saw him, his serum calcium was 7.8 mg. per cent.

We put him on a balance study and, for a 24-hour period, observed his urine phosphorous and calcium excretion.

We then gave him an IV calcium infusion, 15 mgm./kgm., for a 4-hour period: the Howard test. The patient had a rise of serum calcium and a fall in serum phosphorous rather than the rise expected in a normal person. He had an increased excretion of urine phosphorous of more than 60 per cent in a 24-hour period, and he had an inordinate rise in urine calcium excretion, again representing, I believe, the inefficient mechanism. Here is a man with a low serum calcium, and yet his excretion is tremendous; he is unable to retain any excess load of calcium.

This rise in calcium in the serum continued for about 24 hours and then returned toward normal, but the most striking thing is that a normal person, given this load of calcium, would have shown a rise in the serum phosphorous and a tremendous fall in the urine phosphorous. Such excretion is due to short-term cutoff of parathormone excretion.

We were fortunate that this man had not been on any medication for about a year and had no vitamin D therapy because vitamin D persists for many months and makes it difficult to study tubular maximum reabsorption of phosphorous. The normal level for a TMP is between 100 and 150 μmoles per 100 cc. when adjusted to 100 cc. of glomerular filtrate.

This man has the highest level that our renal laboratory had been able to detect in a hypoparathyroid individual, 162 and 163 μmoles of phosphorous per 100 cc. of filtrate when a phosphate load was given to standardize the TMP.

He demonstrates the tubular exhaustion effect, which has been recorded by many patients.

I think we see here another facet of the problem. We are dealing with an endocrine system and a metabolic system that are far from well delineated. As to methods of study, I do not believe that serum calcium level is sufficient to indicate whether a person has parathyroid function.

Many of our animals had normal serum calcium levels; none of them had parathyroid tissue, and all showed inefficient responses to either calcium increase or decrease.

Sterling: In the space allotted for our presentation we could not give an accounting of the various calcium studies that had been made in our cases.

By no means were these patients studied completely as I have had much difficulty in persuading the hospital laboratories to do fecal calcium studies. I have had to be content, in most cases with serum calcium levels and with urinary calcium excretion levels.

We have checked the urinary calcium excretions in all these patients and have demonstrated quite satisfactorily that, prior to transplantation, they did not have adequate calcium excretion no matter what the level of serum calcium was. Following transplantation and until the present time they have had fairly adequate serum levels of calcium and they do show adequate calcium excretion.

AN EVALUATION OF ENDOCRINE TISSUE HOMOTRANSPLANTATION IN THE MILLIPORE CHAMBER: WITH A NOTE ON TISSUE ADAPTATION TO THE HOST*

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INTRODUCTION

Autografts of endocrine tissue in fragment form can obtain nourishment from the host and, in a significant percentage of instances, function and correct a hormone deficiency. Unlike liver or kidney, endocrine tissue needs no immediate vessel-to-vessel connection with the host. Viability of the minute fragments is presumably maintained by a cross-diffusion of nutrients and metabolites.

Histologically and functionally proved permanent homografts of endocrine tissue as yet have not been accomplished in higher animals or man, presumably because of the accepted immunological differences. Reports from various laboratories[1] of successful endocrine tissue homotransplantation in animals of lower orders (mice, rats, and guinea pigs) have led to a confusion of theories. It has been shown by various workers that a degree of success can be obtained with homotransplantation of certain organs, notably the ovary. This fact suggests that endocrine tissues immunologically behave differently from skin or kidney.

Much of the experimental work with endocrine tissue homotransplantation has been carried out on these animlas, using very special sites of transplantation such as the anterior eye chamber and the cerebrum. Here, for special reasons of vascularity, lymph tissue absence, or peculiarity of stroma, endocrine tissues, or skin may survive beyond the accepted time of rejection. In such cases one is dealing with variables pertaining to the host. Much of the experimental work in the past with endocrine tissue "homotransplantation" has been carried out in animals in which close inbreeding has produced an immunological situation equal to or close to an isologous one. In other reports, either confusion has existed with regard to exactly what is meant by homotransplantation or no comment has been made clarifying the degree of genetic dissimilarity of the experimental animal in question.

However, recent carefully controlled work with ovaries in the rat and mouse has shown clearly that there well may be a difference in "antigenicity" between endocrine tissue and skin. Billingham and Parkes[2] have shown that ovarian tissue homotransplanted to rat survived longer than did skin. Parkes has been able orthotopically to transplant ovaries between two inbred strains of mice.[3] Russell and Gittes[4] found a distinct difference between parathyroid and skin in terms of length of survival following transplantation in rats.

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Other workers, on the other hand, failed to obtain significant interstrain takes of ovary,[5] adrenal,[6] or thyroid.[7]

Those who believe that endocrine tissue is more easily homografted theorize in the following manner: (1) endocrine tissue in itself may have different or fewer histocompatibility requirements;[8] (2) the small fragments of transplanted tissue, less likely to become vascularized, fail to come into contact with host cells responsible for the rejection;[9] (3) the recipient's deficiency state establishes hormonal mechanisms that encourage an endocrine "take"; (4) endocrine fragments secure intimate contact with the host more slowly than do other tissues and, in so doing, may initiate a degree of adaptation that allows for prolongation of graft survival.

TABLE 1

HOMOTRANSPLANTATION IN THE SPRAGUE-DAWLEY RAT

Tissue	Total no. transplants	Survival 4 weeks (%)	Survival 10 weeks (%)
Skin	92	1	0
Adrenal (homo- without chamber)	20	5	0
Adrenal (auto- in Millipore)	21	38	38
Adrenal (homo- in Millipore)	36	48	33
Ovary (homo- in Millipore)	70	40	40
Thyroid (homo- in Millipore)	84	70	44

TABLE 2

HOMOTRANSPLANTS IN HUMANS

Organ	Total no. transplants	Histology at 2–4 months (no. transplants)	Function (no. transplants)	
			Immediate	Late 2 months
Thyroid	10	3/10 fair	0	0
Parathyroid	6	1 good	?3	1
Ovary	5	3 good	5	3
Pancreas	2	1 poor	2	?2

To obviate the homograft reaction, we have used the method of Algire,[10] employing the diffusion chamber technique for tissue protection and applying it to the homotransplantation of thyroid, adrenal, parathyroid, and ovary in the rat (TABLE 1), in the dog, and in man (TABLE 2).

MATERIAL AND METHODS*

Donor and Recipient Animals in Experiments

Adult female albino rats of the Sprague-Dawley strain. The rats serving as donors were obtained from the same breeding farm as were the recipients. The donors were younger animals (2 to 3 months old) and both donors and recipients were the products of quite random breeding in a large colony. Attempts to perform skin homografts were made among 92 of these Sprague-Dawley rats. No permanent survival of homotransplanted skin was achieved, although a technique of skin autotransplantation was developed that gave a 98 per cent successful transplantation. The mean survival time of the homografted skin was 13.5 days. In 2 animals the skin rejection was delayed to 22 days; in 1 animal, to 66 days.

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* Detailed reports of *Materials and Methods* and of *Results* may be found in the references.[11-18]

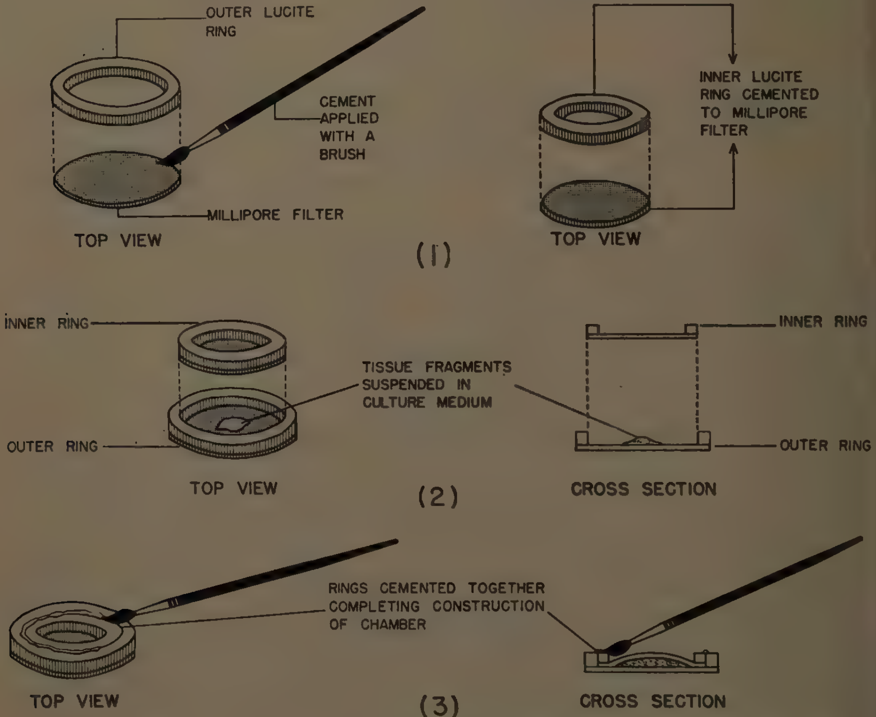
OUTER LUCITE RING
CEMENT APPLIED WITH A BRUSH
MILLIPORE FILTER
TOP VIEW
INNER LUCITE RING CEMENTED TO MILLIPORE FILTER
TOP VIEW
(1)
INNER RING
TISSUE FRAGMENTS SUSPENDED IN CULTURE MEDIUM
OUTER RING
TOP VIEW
(2)
INNER RING
OUTER RING
CROSS SECTION
RINGS CEMENTED TOGETHER COMPLETING CONSTRUCTION OF CHAMBER
TOP VIEW
(3)
CROSS SECTION

FIGURE 1. Steps in construction of the membrane filter chambers used in tissue homotransplantation. Reproduced by permission of *Endocrinology*, March 1960 (*in press*).

Mongrel dogs. Males and females were used without distinction as to donor or recipient.

Humans. Males and females were used without distinction as to donor or recipient.

The Millipore diffusion chambers were constructed by the method of Algire.[10] The chambers utilized the HA Millipore both with and without nylon impregnation. This membrane has an effective pore size of approximately 0.45 μ and is either 100 ± 10 or 150 ± 10 μ thick. FIGURE 1 illustrates the method of assembly of the Lucite rings and Millipore circles into the shallow receptacles that comprise the chamber. The smaller membrane circle (14.5 mm. diameter) fits within the larger (17.5 mm. diameter). The chambers then are sealed with glue, 2 per cent Lucite in acetone, after the tissue is inserted. Seven to

15 mg. of tissue thus are contained in each chamber. The empty chambers are sterilized by exposure to the vapors of ethylene oxide. Sturgis has used slightly larger chambers without peripheral rings.[11]

Adrenal[12,13]

Rats. Bilateral adrenalectomy causes death unless salt therapy is supplied. Accessory adrenals were not encountered. Animals were subjected to transperitoneal bilateral adrenalectomy under open-drop ether and received autotransplanted adrenal tissue (±40 mg.) in 4 chambers placed retroperitoneally. They were maintained on salt therapy thereafter for one month; those animals surviving the first four weeks on salt were the active members of the study. Salt was then removed from the diet of the survivors and the animals were either followed until death or laparotomized at a later date, when the chambers were removed and the animals observed. Serum corticoid levels were not measured. At the time of death the animals were carefully explored for accessory or remnant adrenal tissue. Chambers removed were studied histologically.

A similar protocol was followed for homotransplantation of the adrenal. Other rats underwent homotransplantation of adrenal tissue without chamber protection.

Dogs. Three mongrel dogs were subjected to autotransplantation of adrenal tissue in approximately 25 Millipore chambers in the course of a two-stage adrenalectomy. These animals were given desoxycorticosterone acetate (DOCA) and adrenocorticotropic hormone (ACTH). Serum sodium, potassium, and hydroxycorticoids were followed until death of the animal. The chambers were examined histologically.

Thirteen dogs were subjected to a similar experimental protocol following two-stage adrenal homotransplantation and adrenalectomy.

Ovary[11,14,15]

Rats. Rats were subjected to bilateral oophorectomy and then allowed to stabilize. Homotransplantation then was performed within Millipore chambers intramuscularly, intraperitoneally, and retroperitoneally. Preoperative and postgrafting vaginal cell patterns were studied and, at death, uterine weights were measured. Chambers removed at autopsy were studied histologically.

Monkeys. Ovarian tissue was homografted between castrate females of a *Macacus rhesus* strain and heterografted between monkeys of this and the *Mangueby* strain. Preoperative and postgrafting levels of urinary follicle-stimulating hormone (FSH) were followed and changes in the vaginal smear observed. Progesterone was given to observe withdrawal vaginal flow.

Humans. Five patients with ovarian dysgenesis underwent ovarian tissue homotransplantation from normal donors. Clinical results, urinary FSH levels, and urethral cell smears were observed for an indication of graft function. From 4 patients representative sample chambers were removed for histological study. Transplant sites included the abdominal wall and retroperitoneal pelvic tissues.

Parathyroid

Dogs.[16] Sixteen dogs underwent total thyroparathyroidectomy, in some cases preceded, in others followed, by parathyroid homotransplantation in Millipore chambers. All animals required calcium therapy in the immediate postthyroparathyroidectomy period. After grafting, calcium therapy was gradually discontinued. Serum calcium levels were observed. After a set period of time the chambers were removed and studied histologically, and the clinical course of the animals was followed.

Humans. Six patients with hypoparathyroidism received normal or adenomatous parathyroid tissue in Millipore chambers placed along the abdominal wall, in the groin, and over the pectoral muscles. A total of 15 to 34 chambers was placed in each patient. Each chamber contained approximately 5 to 10 mg. of tissue. Serum calcium and phosphorus levels were followed. Urinary calcium and phosphorus excretion were studied.

Thyroid[17]

Rats. Thyroidectomized rats were given retroperitoneally 2 chambers containing thyroid homografts. At predetermined dates tracer doses of I^{131} were given and, 24 hours later, the chambers were removed, divided, and studied both histologically and from the point of view of radioactivity.

Humans. Ten volunteer males received thyroid tissue as homografts from normal donors. Each chamber was placed in and behind the rectus muscle. At 10 weeks these patients were given a tracer dose of I^{131} and the grafts were removed and evaluated both for radioactivity and histologically.

RESULTS

Adrenal[12,13]

Rats. No animals subjected to total adrenalectomy and adrenal homotransplantation without Millipore protection survived the 4-week control period. Thirty-eight per cent of animals undergoing adrenalectomy and autografting lived 10 weeks or longer. Histological evidence of viable adrenal cells within the chambers gave further corroboration.

Thirty-three per cent of animals undergoing adrenalectomy and homografting lived 10 weeks or longer. Histological evidence of viable adrenal cells within the chambers is noted in FIGURE 2. No grafts survived longer than 6 months.

All chambers, both those with and those without viable adrenal cells, showed extensive fibrosis. Occasional chambers exhibited viable adrenal cells and capillaries with red blood cells concomitantly, suggesting host-graft tolerance (*see below*). No evidence of a leukocytic rejection type of reaction was seen inside these chambers. Leukocytes were commonly noted outside the chambers. On the other hand, no normal endocrine tissue in a broken chamber into which leukocytes had invaded was found.

Dogs. No animals survived beyond the expected length of action of DOCA substitution. Sepsis and fibrosis about and within the chambers were common findings. In the case of 1 animal, there was normal-appearing adrenal

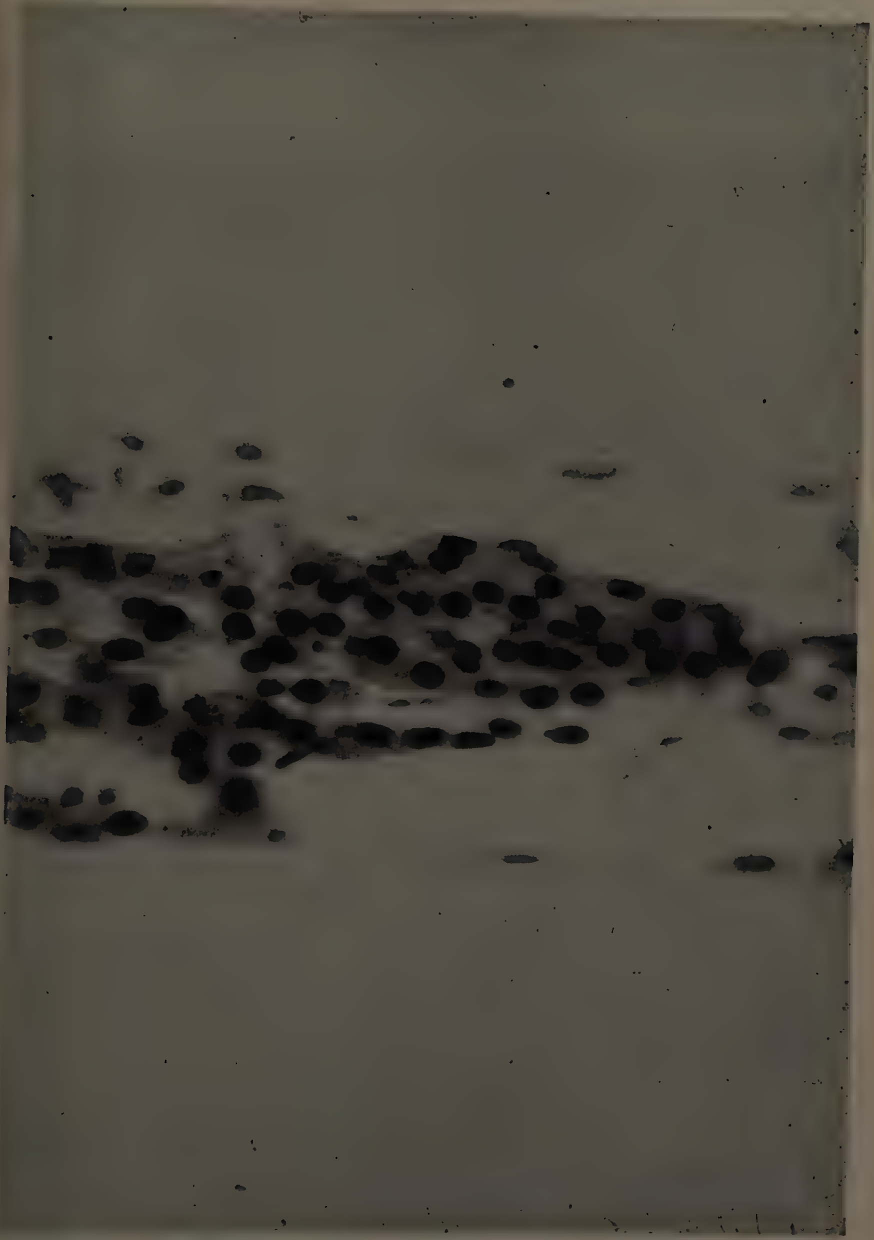

FIGURE 2. Homografted rat adrenal tissue at 6 months with function. Reproduced by permission of The American College of Surgeons from Surgical Forum of the 44th Clinical Congress, **9:** 636, 1958.

tissue 10 weeks after homografting in some of the chambers, but it was not sufficient to maintain the life of the animal (FIGURE 3). At no time were serum hydroxycorticoids at a normal level in any of the animals. There was no appreciable clinical or histological difference between those receiving homo-

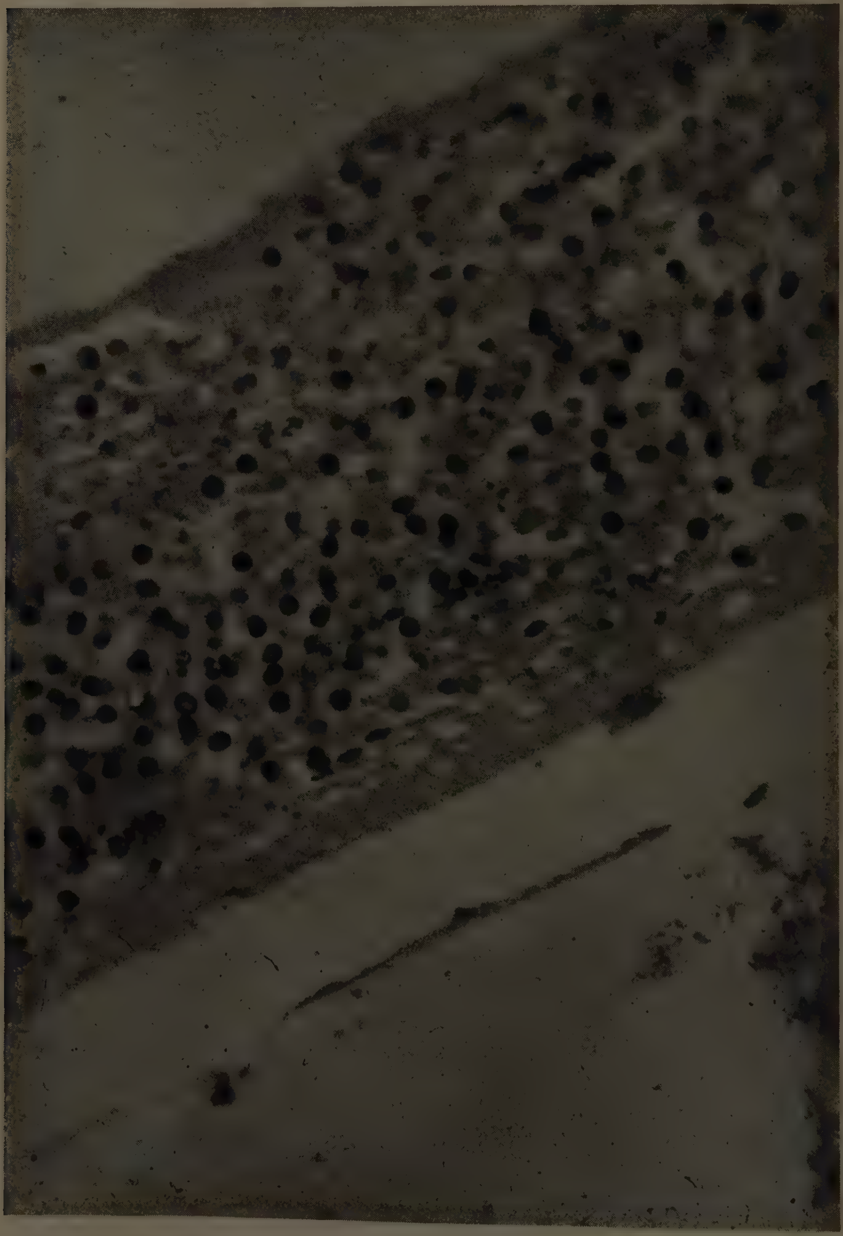

FIGURE 3. Dog adrenal homotransplantation at 10 weeks without function. Scattered adrenal cells and necrotic cells.

grafts and those receiving autografts. The dropping serum sodium levels and rising serum potassium levels routinely preceded death of the animals.

Ovary[11,14,15]

Rats. Forty per cent of the rats exhibited significant estrogen production, sufficient to revert a preoperative abnormal vaginal smear to one that revealed a normal to near-normal estrogen effect. Uterine weights were measured following death of the animal.

The function of these chambers persisted for as long as 6 months in some animals, and removal of the chambers resulted in reversal of the vaginal smear to the castrate type.

Monkeys. In 2 cases homotransplanted ovarian tissue resulted in reversion of the vaginal cell smear to its normal cornification secondary to estrogen activity and produced a drop in FSH levels from the castrate level. In 1 of these monkeys this estrogen effect persisted for 7 months and disappeared with removal of the chambers. Histologically, the transplanted cells appeared viable. There was no evidence of host cells within the chambers.

Humans. Ovarian tissue transplanted to 5 patients with ovarian dysgenesis functioned for from 3 to 7 months, producing a drop in FSH and significant cornification of vaginal cells. Clinical improvement was not significant in any of the cases, and the laboratory findings revealed a reversion to the preoperative state at the end of 6 months. Sample chambers removed at varying times revealed viable ovarian tissue in 3 of the 5 cases. However, no evidence of ovulatory activity was noted.

Parathyroid

Dogs.[16] Adaptation of the parathyroidectomized dog to his deficiency state makes evaluation of parathyroid grafts difficult. Of an original 16 animals, 11 were clinically normal without calcium substitution at the time of Millipore chamber removal. None of these animals appeared to be adversely affected by removal of their Millipore chambers, and there was no significant change in serum calcium following Millipore chamber removal. Microscopic study of the chambers frequently demonstrated apparently viable parathyroid cells lining the membrane, but no chambers contained viable cells in sufficient quantity to support a hypoparathyroid animal. Fibrosis filled the remainder of the chambers studied.

Human. Immediate, extremely short-lived clinical improvement has been seen in 3 of 6 parathyroid chamber homografts, indicative of what might be called the passive transfer of parathormone at the time of grafting. One patient, following homotransplantation of an adenoma, showed improvement for a period of 6 months. At the end of this period of time tests showed very little parathyroid function, and 1 of the 17 chambers, removed as a sample, showed complete fibrosis and no viable parathyroid cells. Another patient not clinically improved had a sample chamber removed at 4 months that showed normal-appearing parathyroid cells (FIGURE 4). In this case apparently insufficient hormone was being produced to replace the deficiency. The other 4 patients have not been improved.

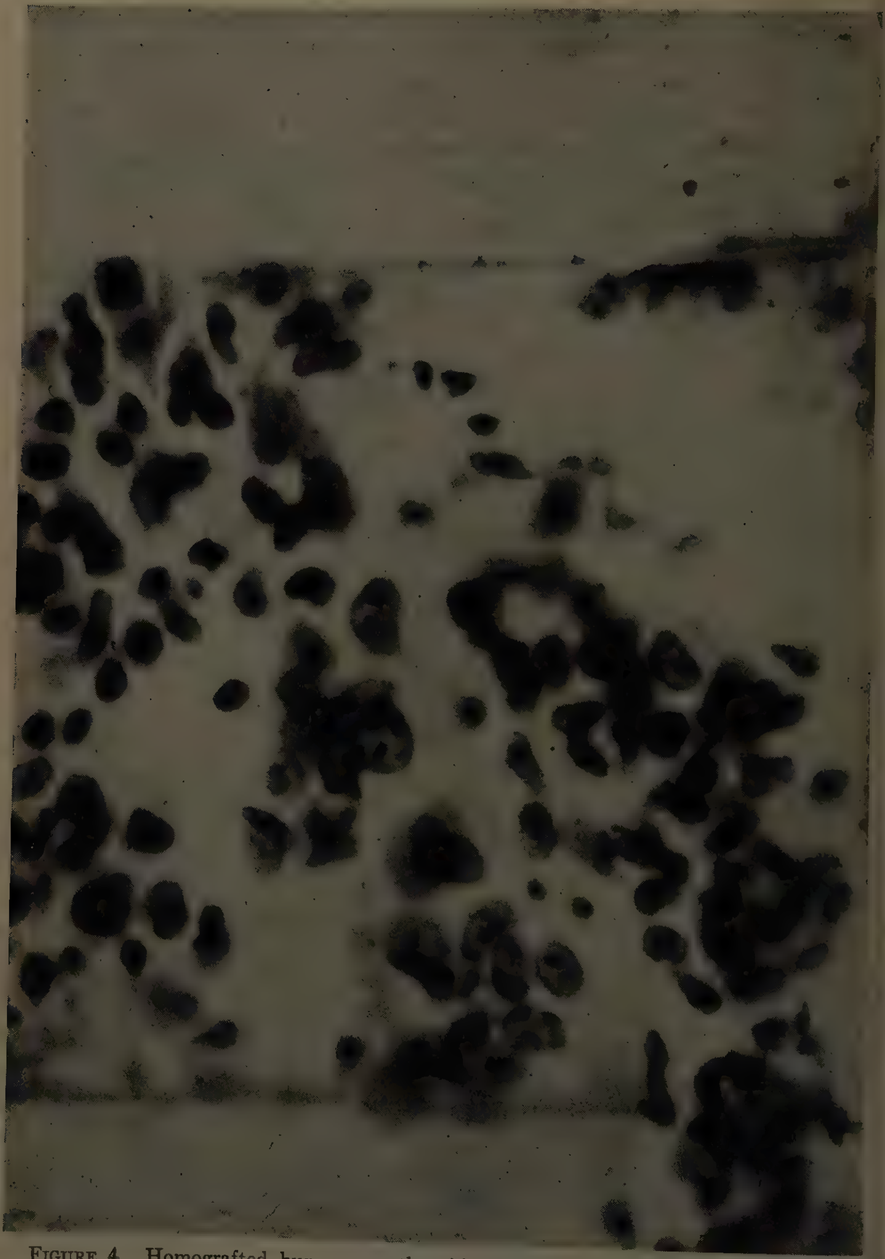

FIGURE 4. Homografted human parathyroid tissue at 5 months. Reinforcing nylon strands can be seen. Reproduced by permission of *American Journal of Surgery*, April 1960 (*in press*).

Pancreas

Human. Two totally diabetic patients received insulinoma-containing chambers. Three months postgrafting, both patients are maintaining a 50 per cent reduction in their insulin requirements. One chamber removed as a sample from 1 of these patients reveals no viable pancreatic tissue. The remaining 23 chambers have not been disturbed.

Thyroid[13,17]

Rat. Seventy per cent of thyroid homografts survived and functioned for 2 months after grafting. Forty-four per cent survived and concentrated I^{131} for 4 months (FIGURE 5). Thereafter a steady decline in apparent tissue viability occurred. It was noted that 16 per cent of thyroid-containing chambers also contained red blood cells and capillaries (FIGURE 6; see under *Discussion*).

Human. In man, at 10 weeks after thyroid homotransplantation, normal thyroid follicles were seen in 3 of 10 cases (FIGURE 7). However, there was considerable fibrosis, but no evidence of characteristic rejection within the chamber. None of these chambers concentrated a significant amount of the radioactive iodine.

DISCUSSION

Short-term (2 to 5 months) successful takes of endocrine tissue within the Millipore chamber appear to be attainable in a significant number of instances. Failure to achieve greater longevity appears to be related to failure of metabolism rather than late rejection.[19] Histologically, the late death of tissue within the chambers is not associated with the typical cellular pattern of rejection. Characteristically, fibrosis has occurred within the chamber and has been interpreted as being fibroplasia or overgrowth of stromal elements at the expense of epithelial elements. In such cases, unless a frank break in the chamber has occurred, host lymphocytes or monocytes or polymorphonuclear leukocytes are seen rarely. The histological picture outside the chamber has been one of granulation tissue and foreign-body reaction. Within the limits of possible interpretation, it appears that this work further substantiates the original homotransplantation concepts of Algire[10] of a cell-bound antibody rather than a freely circulating one. If "late rejection" is the cause of our failures, then it must be associated with a cellular response different from that commonly attributed to the early rejection phenomenon.

Our work and that of others with the Sprague-Dawley strain of rat suggests that endocrine tissues are not all equally subject to classical homotransplant laws. Unprotected adrenal and probably thyroid homografts are rejected in a standard manner, but parathyroid[4] and ovarian tissues appear to have a degree of histocompatibility that allows a certain percentage of unprotected homografts to survive.

The interesting concomitant findings of endocrine tissue and of capillaries containing erythrocytes within the Millipore chambers in rat thyroid (FIGURE 6) (16 per cent) and adrenal homotransplants raise the question of tissue adaptation to the host.[7] It seems unlikely that the capillaries and cells are developed from vascular elements in the donor tissue, for primitive erythro-

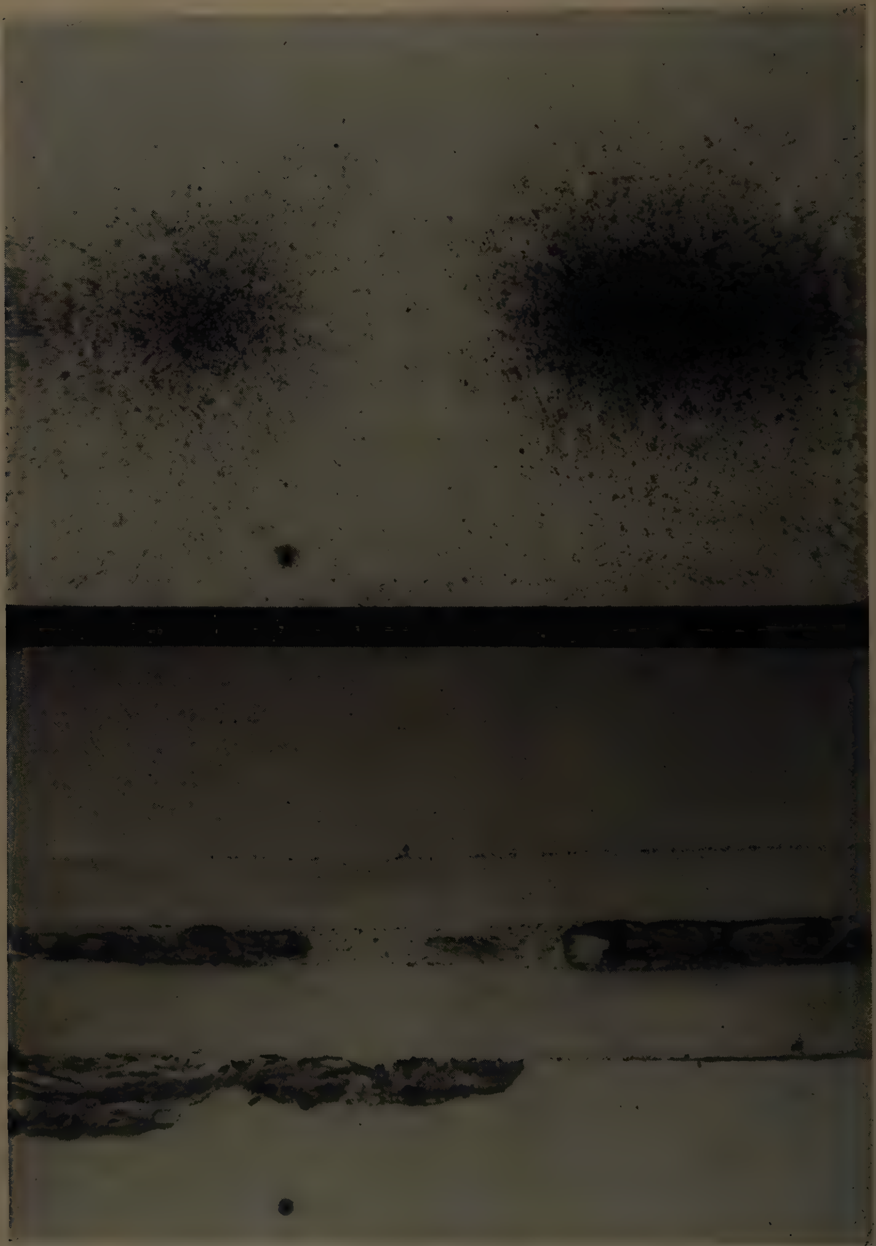

Figure 5. Microautoradiograph and corresponding tissue section, showing localization of radioactivity in follicular areas of homotransplanted rat tissue. Homotransplant within HA filter chamber was removed 2 months after implanting. ×36. Reproduced by permission of *Endocrinology*, March 1960 (*in press*).

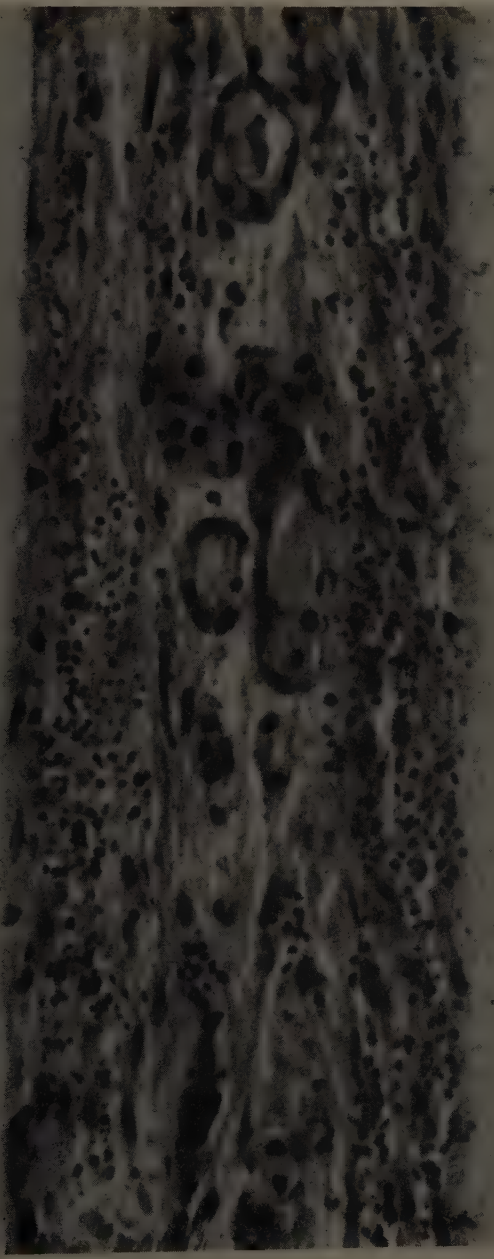

FIGURE 6. Diffusion chamber containing homografted thyroid tissue, removed 1 month after implantation and showing erythrocytes in capillaries surrounding histologically identifiable thyroid tissue. ×100. Reproduced by permission of *Endocrinology*, March 1960 (*in press*).

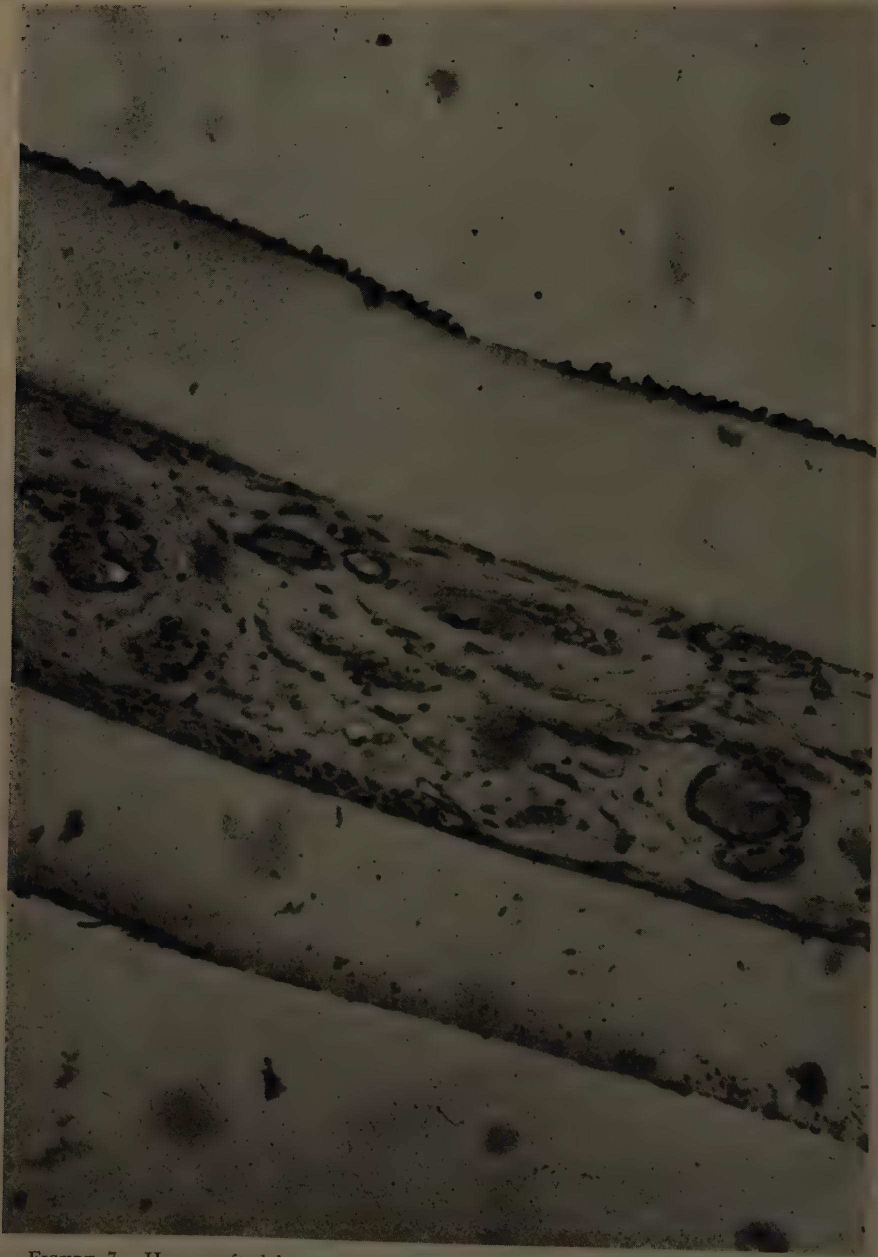

FIGURE 7. Homografted human thyroid at 10 weeks. Note fibrosis. Reproduced by permission of *American Journal of Surgery*, April 1960 (*in press*).

cytes or leukocytes never have been seen within the chamber. Therefore, the presence of capillaries is strong evidence that the chamber has been broken and that host tissue is in contact with the grafted tissue. The fact that there is usually no inflammatory response in such cases, either leukocytic or lymphocytic, suggests that the host is tolerant of the grafted tissue. It is conceivable that transplantation within the Millipore chamber in these cases has acted in some manner as a "conditioning" influence.

At the moment the fate of the Millipore technique appears to rest upon a solution to the problems of faulty tissue metabolism rather than on immunology. The membrane itself is a barrier to diffusion[19] and, as a result, extensive fibrosis within the chamber is a common finding. Few chambers fail to show at least some fibrosis and resulting loss of epithelial cells. Even "normal-appearing cells" in chambers with minimal fibrosis may not be always "normally functioning cells." Certainly, with the passage of time this becomes more true.

REFERENCES

1. KROHN, P. L. 1959. Endocrine tissue transplantation. *In* Transplantation of Tissues. : 409–470. Williams & Wilkins. Baltimore, Md.
2. BILLINGHAM, R. E. & A. S. PARKES. 1955. Studies on the survival of homografts of skin and ovarian tissue in rats. Proc. Roy. Soc. London. **B143:** 550.
3. PARKES, A. S. 1956. Survival time of ovarian homografts in two strains of rats. J. Endocrinol. **13:** 201.
4. RUSSELL, P. S. & R. F. GITTES. 1959. Parathyroid transplants in rats. A comparison of their survival time with that of skin grafts. J. Exptl. Med. **109:** 571.
5. KROHN, P. L. & D. L. INGRAM. 1956. Factors influencing the survival of ovarian homografts in rats. J. Endocrinol. **14:** 110.
6. MEDAWAR, P. B. & P. S. RUSSELL. 1958. Adrenal homografts in mice with special reference to immunological adrenalectomy. Immunology. **1**(1): 1.
7. WOODRUFF, M. F. A. 1959. Evidence of adaptation in homografts of normal tissue. *In* Biological Problems of Grafting : 82–94. Blackwell Sci. Publ. Oxford, England.
8. KROHN, P. L. 1959. Ovarian transplantation in mice. *In* Biological Problems of Grafting. : 146-162. Blackwell Sci. Publ. Oxford, England.
9. MERWIN, R. M. & E. L. HILL. 1954. The fate of vascularized and nonvascularized subcutaneous homografts in mice. J. Natl. Cancer Inst. **14:** 819.
10. ALGIRE, G. H. 1954. Growth of cells *in vivo* in diffusion chambers. I. Survival of homografts in immunized mice. J. Natl. Cancer Inst. **15:** 493.
11. STURGIS, S. H. & H. CASTELLANOS. 1958. Ovarian homografts in the primate: experience with Millipore filter chambers. Am. J. Obstet. Gynecol. **76:** 1132.
12. BROOKS, J. R., J. C. PRIARIO & A. DE SCOVILLE. 1959. Endocrine tissue homotransplantation. *In* Surg. Forum, Proc. 44th Congr. Am. Coll. Surgeons **9:** 633. Am. College of Surgeons. Chicago, Ill.
13. STURGIS, S. H. & H. CASTELLANOS. 1957. Functional survival of ovarian homografts within Millipore filter chambers in the castrate rat. Proc. Soc. Exptl. Biol. Med. **94:** 569.
14. CASTELLANOS, H. & S. H. STURGIS. 1958. Ovarian homografts. Obstet. Gynecol. **12:** 603.
15. WILSON, R. E., R. M. ZOLLINGER, JR., J. H. MAHAN & J. R. BROOKS. 1959. Homotransplantation of canine parathyroid tissue in Millipore diffusion chambers; the phenomenon of adaptation to the aparathyroid state. Presented to the 45th Clin. Congr. Am. College of Surgeons.
16. BROOKS, J. R., G. J. HILL, III, A. DE SCOVILLE, J. C. PRIARIO, D. CROCKER & H. A. SELENKOW. 1960. A study of the survival and function of homografted thyroid tissue in membrane filter chambers in the rat. Endocrinol. **66:** 392.
17. BROOKS, J. R. & G. J. HILL, II. 1960. Current status of endocrine homografts using the Millipore diffusion chamber technique. Am. J. Surg. In press.
18. HILL, G. J., II, J. R. BORDER, A. SICULAR, J. R. BROOKS, & F. D. MOORE. 1959. Investigations into the properties of membrane filters used in tissue homotransplantation. Presented to the 45th Clin. Congr. Am. College of Surgeons.

Discussion of the Paper

Paul S. Russell: First, with respect to Brook's paper: in the course of some experiments designed for another purpose, we have had occasion to assess the viability of sheets of pure epidermal cells maintained in filter chambers made according to the standard method described by Algire. These sheets were prepared by trypsin digestion from grafts cut from a dorsum of a rabbit's ear. They were then sealed into the chamber and placed in a pocket beneath the skin of the chest on the surface of the panniculus carnosus muscle.

These chambers were made precisely according to Algire's original description, and they were composed of filter material, 150 μ thick, with a pore size of 0.45 μ.

Three to four weeks later, the chambers were removed, and their contents were seeded onto a bare area prepared on the original donor rabbit's chest.

One could see on that bare area a nice layer of epidermal cells whose presence was confirmed histologically. This occurred in about 40 per cent of the trials. It demonstrated to us that normal cells could survive under these circumstances well beyond the period to be expected in the unprotected state.

About two years ago, we attempted transplantation of adrenocortical tissue between strains of mice, placing the Algire chambers into the peritoneum and retroperitoneum of adrenalectomized recipients according to an experimental design that Medawar and I had employed previously in studying unprotected adrenal grafts.

We had no convincing success, as adjudged by the ability of these chambers containing adrenal tissue to sustain life, in spite of the fact that on occasion we could find histological evidence of survival of fragments of glandular tissue within the chambers. We had a similar experience in the rat, using parathyroid tissue. Here we made use of the serum calcium level. The parathyroidectomized recipient was maintained on a very low calcium diet, as a measure of hormone activity.

We saw the same type of fibrous tissue proliferation within the chambers that Brooks has described, and we felt that the basic difficulty was probably explained on a nutritional basis, since no better results were obtained in autologous transfers.

We therefore varied the dose of tissue within the chamber within wide limits and designed chambers using filter material as little as 30 μ in thickness, with no better results.

I think Brooks and his colleagues deserve congratulations for their persistence along this avenue of technical frustration, since there is no theoretical reason why they should not have an increasing success.

With respect to Sterling's contribution: we have had considerable experience in the rat with parathyroid homografting. I believe that we can say, at least in regard to our own animals, that parathyroid tissue is clearly different from skin in its complement of antigens. It is deficient in antigens with respect to, and in comparison to, skin.

I make no statement about other animals, or even about other rats because, I dare say, they will or might be different. To date, the deficiencies of antigens in endocrine tissue with respect to other tissues have been observed only in the rat.

I think the theoretical importance of these observations is that tissues can differ. From this experience of ours, I believe that it is not yet proved that Sterling's results, in so far as they indicate success (and I think it very difficult to assess this), may well be on the basis of the fact that his tissues used for transplantation were parathyroid rather than skin.

I agree with Wilson that the metabolic evidence is confusing and difficult to evaluate. I think an adaptation may well occur, but I do not believe that we can yet exclude the presence of small islands of the host's own accessory tissue that gradually hypertrophy and take over a portion of the original function of the intact gland.

I agree with Conway that what we need is histological proof. Nothing else will be really satisfactory.

C. A. L. Bassett (*Columbia University College of Physicians and Surgeons, New York, N. Y.*): I wonder whether the rejection that we are observing in these tissues after a long period of time can be assigned to a late rejection phenomenon. I say this because the maintenance of viability within these chambers seems to be dependent largely upon the flow-rate characteristics of the Millipore itself.

In some studies that Campbell and I have been making on the regeneration of peripheral and central nervous system tissue within Millipore chambers for the past four or five years, we have been impressed by the absolutely essential feature of maintaining a high rate of flow through these chamber walls. We have been amazed to find directly that, with the passage of time, the Millipore becomes progressively plugged with calcium phosphate deposits, so that our flow rate begins to drop off precipitously about 2½ to 3 months after implantation.

I suspect that this may possibly provide us with some answer about late failures.

George L. Jordan, Jr. (*Cora and Webb Mading Department of Surgery, Baylor University College of Medicine, Houston, Texas*): We have been interested primarily in the problem of parathyroid transplantation and have been investigating this in the dog and in the human. We have done studies in the rat that are very similar to those reported by Russell and we have arrived at a similar conclusion: namely, that the parathyroid gland is very different from any of the other distinct tissues.

We have observed, first, that transplants of parathyroid glands are more likely to survive for long periods of time than other tissues that we have transplanted.

Second, we have found that the transplantation of parathyroid gland does not produce transplantation immunity when tested with subsequent skin transplants. Third, we have some evidence to suggest (although we do not regard the evidence as unequivocal), that the hypoparathyroid state itself may alter the ability of the host to respond to transplanted tissue in the rat, for example, when tested with a skin transplant.

I have been very much interested in the work of Sterling; we have performed two total thyroid-parathyroid transplants, using techniques similar to those he has described. Neither of our patients has had a return to perfectly normal parathyroid function. Both of these patients, however, are significantly better than they were preoperatively. Both had been hypoparathyroid for years—that is, 4 and 6 years respectively.

After our first transplant, performed in a man who previously had had a pretransplant of parathyroid adenoma without any evidence of function whatsoever, the serum calcium rose. Not only did it rise to normal levels; it rose to abnormal levels. It rose to 14 mg. percent in the first few days following surgery. This certainly suggested that there was something there more than just a psychological effect or other adaptive mechanism.

This man is now three years postoperation. In his preoperative state, he was taking orally 24 gm. of calcium, 4 tablets of dihydrotachysterol, and 100,000 units of vitamin D a day. With this maintenance supportive therapy, he could maintain a normal serum calcium; without it, he could not.

For the past three postoperative years he has still taken some calcium, about 8 to 12 gm. of calcium a day. He has not taken any vitamin D. He has not taken tachysterol and, during this time, he has maintained a normal serum calcium and has remained asymptomatic. Our second patient has had a similar course.

We have also investigated transplantation using parathyroid adenoma grown in tissue culture, and we have now performed 12 such transplants in a total of 6 patients. None of these transplants has shown the pattern that we observed with the total thyroid-parathyroid transplants.

Following several adenoma transplants, patients have had a rise of serum calcium and have experienced an improvement in symptoms; then, after a period of about two months, their serum calcium fell to low levels and symptoms returned.

We have one patient whom we have now transplanted with cultured parathyroid adenoma tissue four times. She has followed the cycle described above four times; that is, she has been better immediately after a transplant and then has had a return to significantly hypoparathyroid levels with return of symptoms and with a low serum calcium.

Therefore, in so far as our clinical work is concerned, the total transplants certainly appear to have behaved differently from the free transplants of cultured tissue.

MACKEE: For some time we have been studying, in a more direct approach, the function of adrenal autotransplants in the rat, both in chambers and outside of chambers. We have been specifically interested in measuring the steroid hormone production of these transplants. In our chambers, which are somewhat different than those used by Brooks and his group, and which we have modified to permit removal of fluid for biochemical analysis, we have run into essentially the same problems as Brooks has. We find the same cytological picture. We have the same level of survival, about 35 per cent in homotransplants and about 33 per cent in autotransplants.

One wonders whether Brooks believes that the methods by which tissues are introduced into the chamber make any difference. We have some preliminary findings that suggest strongly that this might be, in effect, an important factor.

Tissue that is finely minced and introduced into the chamber seems to take better, providing a higher percentage of survival than when the tissue is introduced in the form of halves, thirds, or quarters.

BROOKS: In answer to Mackee: we believe that the tissue fragments should be as small as possible and, of course, we feel a very important aspect is the proper handling of these fragments.

ELTON WATKINS (*Lahey Clinic, Boston, Mass.*): Although I certainly agree with what Conway has said, I am somewhat more enthusiastic than he and somewhat less so than Sterling.

At the present time we are using the aortic pedicle technique that we have described, which is merely a variation of Sterling's pioneer contributions. We remove both carotids along with the aorta and this, consequently, provides a somewhat larger vessel to anastomose. The youngest fetus we have used for donor material was in the sixteenth week of gestation. In the latter donor material, we made an anastomosis between the aortic arch of the pedicle and the cut end of the inferior deep epigastric artery. It illustrated that it was possible to perfect this type of anastomosis with 7-0 silk.

We have now made seven such pedicle transplants, including the one first performed in 1955. A further follow-up study confirms what Wilson has reported. As a surgeon, I am abashed to say that this patient developed a hernia at the transplantation site five years later, and fifteen biopsies from the pedicle merely demonstrated the presence of granulation tissue, without any demonstration of viable parathyroid tissue.

This problem, I think, is a little difficult because, as others have mentioned, the only true criterion of demonstrating lack of viability would be an entire excision of the pedicle, a step which we are extremely reluctant to take in any of these patients.

Of the other six patients operated upon in the last year, two are no longer on supportive therapy and the other four have a therapy reduced to levels below that which produced tetany under hospital conditions. Therefore, I too believe that our experience, as it is shaping up, infers that there is an apparent late reduction of viability of grafts, but an early improvement that can be documented by chemical changes.

ROGERS: It might be interesting to Watkins to know that there are several inadvertent precedents to his last suggestion, namely, the unfortunate removal of a homotransplanted endocrine gland resulting in a human patient's return to his original preoperative endocrine deficiency.

As a demonstration that fetal endocrine tissues survive, function, and correct the endocrine deficiency in human patients, Greene (Greene, H.S.N., report read at the Colloques International: La Biologie des Homogreffes, Paris, 1957) described the successful treatment of a patient with Addison's disease by the transplantation of a fetal adrenal gland placed in the patient's rectal abdominal muscle. Prior to transplantation the patient required corticosteroid therapy and DOCA implantation but, subsequent to transplantation, the patient no longer needed this hormonal therapy. Interestingly, in subsequent months the patient reported to a local surgeon for a lump in the abdomen in the region of the site of transplantation and the surgeon unknowingly, not familiar with the previous transplantation, excised the lump and, in doing so, apparently removed the fetal adrenal tissue that had been transplanted. As a result, the patient again went into an Addisonian state and required resumption of the hormonal therapy.

THE DORMANT STATE IN TISSUE TRANSPLANTATION AS EXEMPLIFIED BY SUBCAPSULAR GRAFTS OF TESTICLES FROM HYPOPHYSECTOMIZED MICE

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Introduction

It is well known, as pointed out in a review of this matter (May, 1932), that mammalian infantile testicular tissue can be transplanted, while adult testicular tissue cannot. It appears (May, 1938) that gonadal tissues remain up to puberty in a state similar to that of the fetal period. This is the apparent reason for the fact that during this embryonic dormant state the genital glands may be transplanted without arousing reactions from the adult host; this condition, observed only in embryonic or very young tissues, is the essential basis of brephoplastic transplantation (May, 1952).

The passing of the glands from the infantile dormant state of reproduction to their adult active functional state of puberty occurs in all likehood under the influence of the gonadotropic hormones. Indeed, it is well known that hypophysectomy in young mammals completely inhibits the growth of their gonads and maintains the latter in a permanent state of atrophy.

I showed in 1955 that, in the case of the mouse, brephoplastic transplantation of a hypophysis to under the tunica albuginea of a testicle rendered atrophic by hypophysectomy brings about the development of its tubuli spermatogenesis and the growth of its interstitial tissue (May, 1955). This local action of the hypophyseal gonadotropic hormones on the testicles of hypophysectomized mice completes the results of experimentation by Max Aron and by his collaborators, especially Petrovic, conducted since 1953 on nonhypophysectomized animals. The elegant experiments of these authors showed that the testicles of normal adult guinea pigs tolerate the transplantation of adult hypophyseal and thyroidal tissues, which would be impossible for other organs. In these normal testicles the gonadostimulins from the transplanted hypophyseal tissue bring about a strong stimulation of the interstitial gland and the inhibition of spermatogenesis, all the greater as one considers the zones nearest the transplanted hypophysis.

In our case, however, the atrophic testicles of a mouse hypophysectomized while still very young appear to remain permanently in a dormant state similar to that in which they were before puberty. This permanently dormant state, however, can be interrupted by the local action of a hypophysis that is transplanted while still in the embryonic state and that grows in its new testicular site.

The question arose as to whether such dormant testicles could not be subjected to the global, and not merely local, action of the hypophyseal hormones, this being attained through their transplantation into another mouse bearing its hypophysis.

I showed in 1939 that the subcapsular site of the kidney lends itself particularly well to testicular transplantation; for this reason I chose it for the experiments described in this paper.

Methods

Young male albino mice weighing from 8 to 12 gm. were hypophysectomized by the method I described in 1953. One month after this operation the mice were weighed and their testicles inspected for atrophy. If they were atrophic, the mice were kept alive another 3 months. After this lapse of time and a further weighing, the 2 testicles were excised. The right one, which was to serve as a control, was fixed in Zenker's fluid. The left one was grafted *in toto* under the capsule of the right kidney of a male albino mouse of the same strain, weighing 13 gm., that had been bilaterally castrated just before the testicular graft.

One or two months later the host mouse was sacrificed and its right kidney studied macroscopically and then fixed in Zenker's fluid.

The right control testicle and the portion of kidney that bore the left testicle were sectioned 7 μ thick and stained with hemalin-erythrosin. I was thus able to compare histologically the right testicle from a mouse hypophysectomized 4 months previously with its left testicle, which furthermore had been transplanted and left for 1 or 2 months in a castrated mouse bearing its normal hypophysis.

In addition, I have made several series of control experiments:

1. I transplanted under the kidney capsule of male adult mice, bilaterally castrated, a testicle from a newborn mouse. One month later the host kidney was excised and treated as described above.
2. I grafted adult testicles to adult male bilaterally castrated mice, but as adult testicles are too large to be placed under a kidney capsule, we were forced to place them in the retrorenal region of the coelom. One month later these testicles were fixed and sectioned as described above.
3. Young mice were hypophysectomized as described above. One month later the right testicle was excised and fixed. The mice then received intraperitoneally on 3 consecutive days 0.1 U. of rat gonadotropic hormone (Choay). On the fourth day the mice were sacrificed and their left testicles fixed and compared macroscopically and histologically with their own right control testicles.

Experimental Results

Figures 1 and 2 (*right*) show, at low and higher powers, sections of the right testicle of mouse B XX, 1, made 133 days after hypophysectomy. One may observe the atrophic aspect of the tubuli, whose diameters average 50 μ, and the very feeble development of the interstitial tissue (figure 1). At a high power (figure 2, *right*) one may see that the cellular layer nearest the tubular basement membrane is constituted essentially of Sertoli's cells; one may also observe a certain number of spermatogonia, although less than in the normal testicle. Toward the lumen is a restricted number of spermatocytes. There are neither spermatids nor spermatozoa.

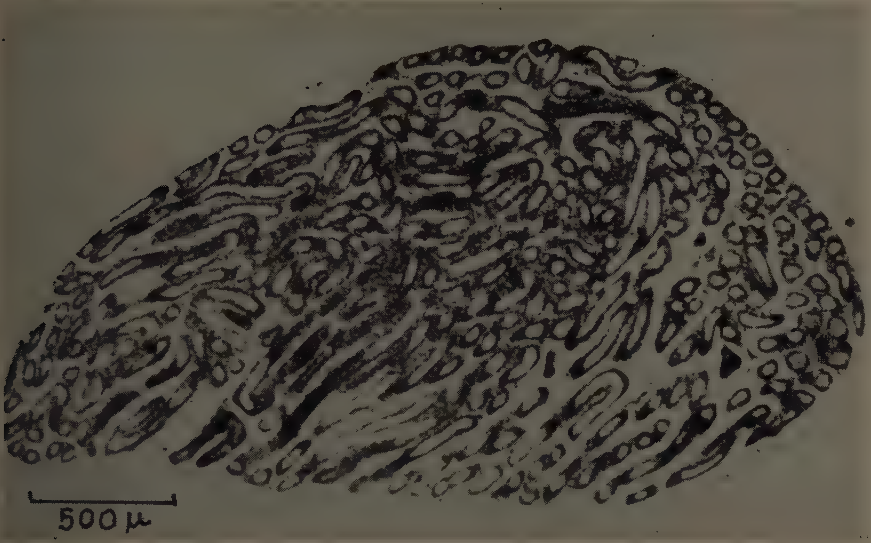

FIGURE 1. Mouse B XX, 1. Right testicle fixed 133 days after hypophysectomy; atrophic aspect.

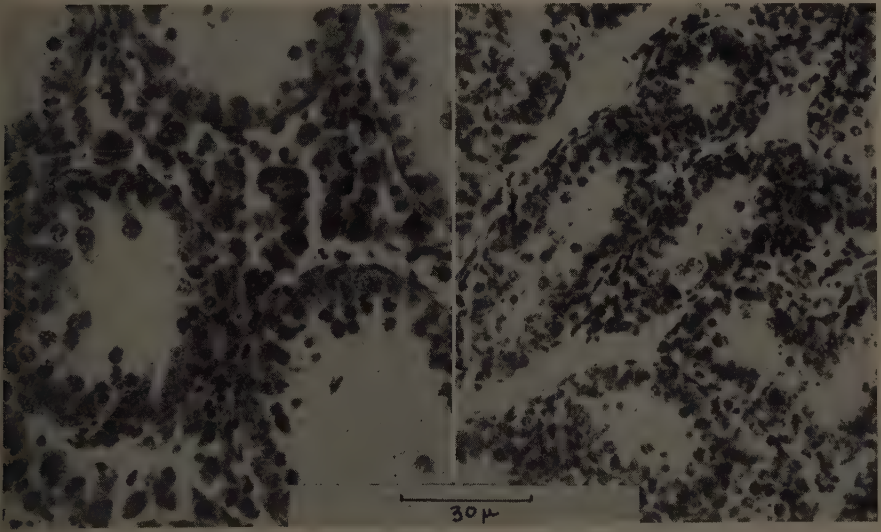

FIGURE 2. Mouse B XX, 1. *Right,* right testicle fixed 133 days after hypophysectomy; atrophic aspect of the tubuli, which contain chiefly Sertolis' cells; very slight development of the interstitial tissue. *Left,* left testicle fixed 34 days after its transplantation under the kidney capsule of a male bilaterally castrated adult mouse, the graft having been made 133 days after hypophysectomy of the donor; the tubuli and the diastematic interstitial tissue are far more developed than those of the right control testicle.

FIGURE 3 shows at lower power a section of the left testicle of the same mouse, under the kidney capsule where it had been implanted 34 days previously, the same day on which the right testicle (FIGURE 2, *right*) was excised and fixed. When the castrated host mouse was sacrificed we found this left testicle under the kidney capsule, where it had been placed, whitish in appearance but well vascularized and forming a round eminence about 4 mm. in diameter.

The section shown in FIGURE 3 reveals that the central part of the transplanted testicle is constituted of seminiferous tubules that are very much atrophied. However, they are well developed at its periphery, especially in the region near the kidney, from which arise the testicle's blood vessels. There the tubuli have a diameter of 80 to 100 μ. Near their basement membrane are still a goodly number of Sertoli's cells. A very great number of spermatogonia can also be seen. Near the lumen are very numerous spermatocytes (FIGURE 2, *left*).

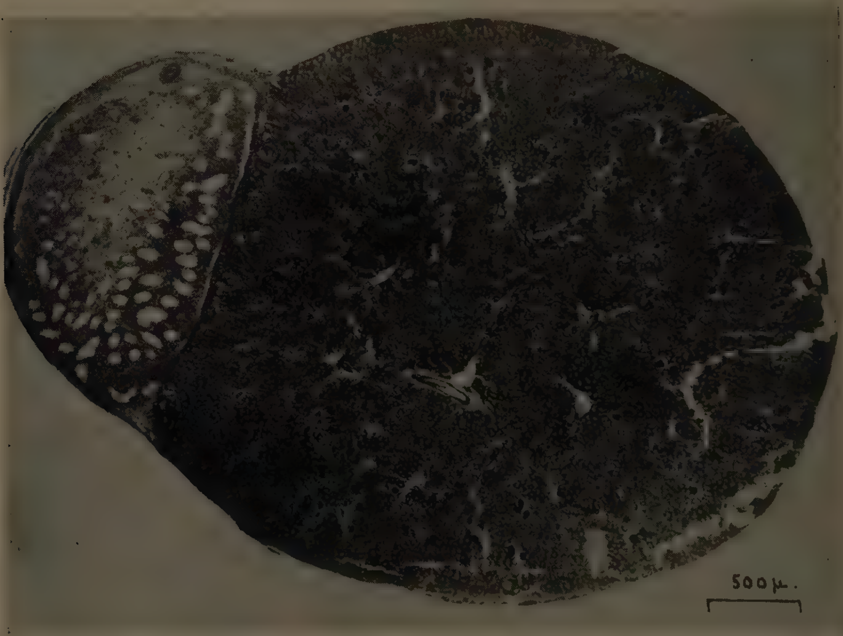

FIGURE 3. Mouse B XXI, 1. Low-power magnification of the left testicle of mouse B XX, 1 (FIGURE 2) *in situ* under the kidney capsule.

Between the tubuli, on the entire periphery of the graft, may be observed an enormous mass of large interstitial diastematic cells (FIGURE 2, *left*). These cells are well developed and occupy the entire space between the tubuli, which they even seem to force back. It is only on the periphery of the transplanted testicle, especially near the kidney, that some clumps of immigrated cells, lymphocytic in type, are observed.

Our other cases, 7 in all, are very similar. In another very typical case, that of mouse B XXII, 1, the right testicle, much atrophied, again is constituted of tubuli very much reduced in size in which Sertoli's cells dominate, and of interstitial tissue that also is much reduced (FIGURE 4, *right*). The left testicle of the same mouse, however, 2 months after its transplantation under the kidney capsule, is well developed: about 5 mm. in length and 4 mm. in width. One may see the tubuli *in situ* with a low-power lens. Histologically, these ducts are much larger than the control tubuli of the right testicle. Spermatogonia and spermatocytes dominate, with an interstitial diastematic tissue that is greatly developed (FIGURE 4, *left*).

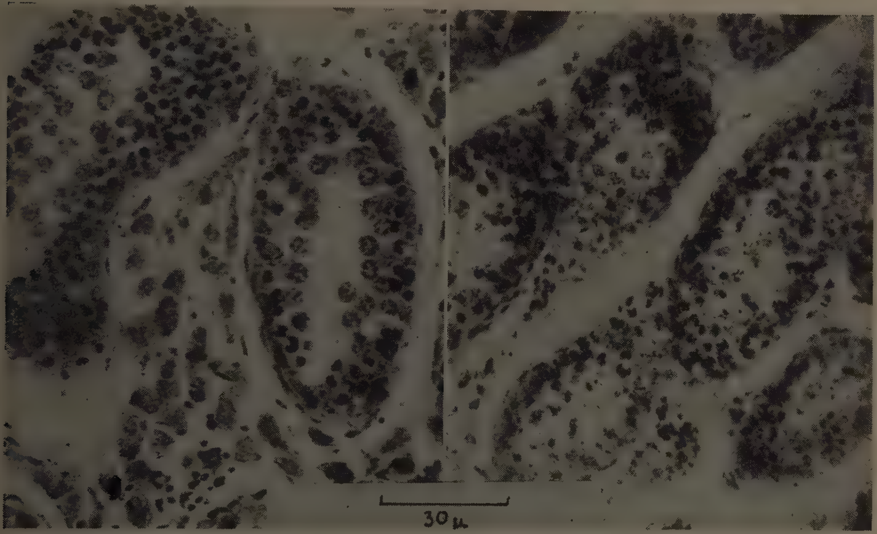

FIGURE 4. Mouse B XXII, 1. On the right, right testicle fixed 123 days after hypophysectomy. Atrophic aspect of the tubuli; very small development of the interstitial tissue. On the left, left testicle fixed 61 days after its transplantation under the kidney capsule of a male bilaterally castrated adult mouse, the graft having been made 123 days after hypophysectomy of the donor; the tubuli and the diastematic interstitial tissue are far more developed than those of the right control testicle.

Thus if we compare, in FIGURES 2 and 4, the right testicle (*right*) and the left testicle (*left*) at the same magnification, we see how different are these two testicles from the same mouse. Following a long period of hypophysectomy begun in the infantile period and lasting 4 months, such testicles are very much atrophied: their tubuli, small in diameter, contain mostly Sertoli's cells, and the intertubular spaces show very few small interstitial cells.

After a 2 months' stay under the kidney capsule of a castrated adult mouse bearing its hypophysis, the grafted testicles show tubuli that have about doubled in diameter and contain many more spermatogonia and spermatocytes. Their interstitial tissue is constituted of an enormous number of large diastematic cells.

Let us now compare such transplants with those of testicles taken from newborn mice and placed under the kidney capsule of bilaterally castrated adult male mice. FIGURES 5 and 6 show specimens from 3 of the 6 animals on which we have operated in this way.

FIGURE 5 (B XXIX, 1) shows a testicle 36 days after its transplantation. At that time it formed a whitish round eminence under the kidney capsule, about 3 mm. in diameter. At a low power (FIGURE 5) the tubuli are seen to be between 70 and 80 μ in diameter. A basal layer is formed by cells of the spermatogonial type, all quiescent and showing no evolution toward the spermatocyte stage. Apart from this layer, the tubuli are almost empty of spermatogenic cells and therefore show large lumens. Between the ducts the basophilic tissue contains but few active diastematic-type cells.

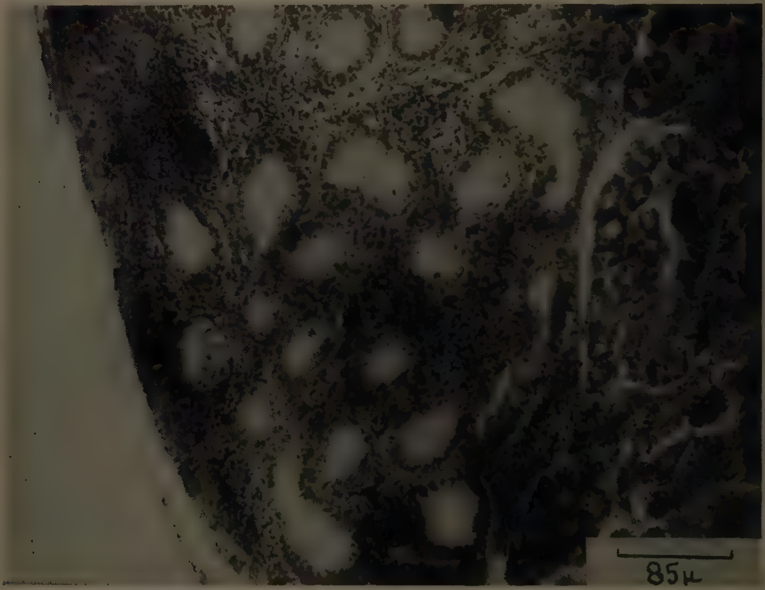

FIGURE 5. Mouse B XXIX, 1. Testicular graft from a newborn mouse, fixed 36 days after its transplantation under the kidney capsule of a male bilaterally castrated adult mouse. Small development of the tubuli and the interstitial diastematic tissue.

Other such cases of newborn testicles transplanted for only 21 days under the kidney of a male bilaterally castrated mouse are shown in FIGURE 6 (mouse B XXXI, 5′ at a low power and mouse B XXXI, 5 at a higher magnification). In both these cases the graft was well vascularized under the kidney capsule, but in the first case (B XXXI, 5′) there occurred a rather important lymphocytic infiltration. Some of the tubuli are well provided with cells, but a certain number of these are pyknotic and degraded (FIGURE 6*a*). The small diastematic cells are relatively few in number. In the other case (B XXXI, 5) the tubuli are light in their cellular content and in their appearance (FIGURE 6*b*) and show spermatogonial-type cells but no mitotic activity and no other type of germinative elements. Here the interstitial cells are more abundant.

As opposed to these transplanted testicles, which came from hypophysectomized or from newborn mice and achieved a certain development under the

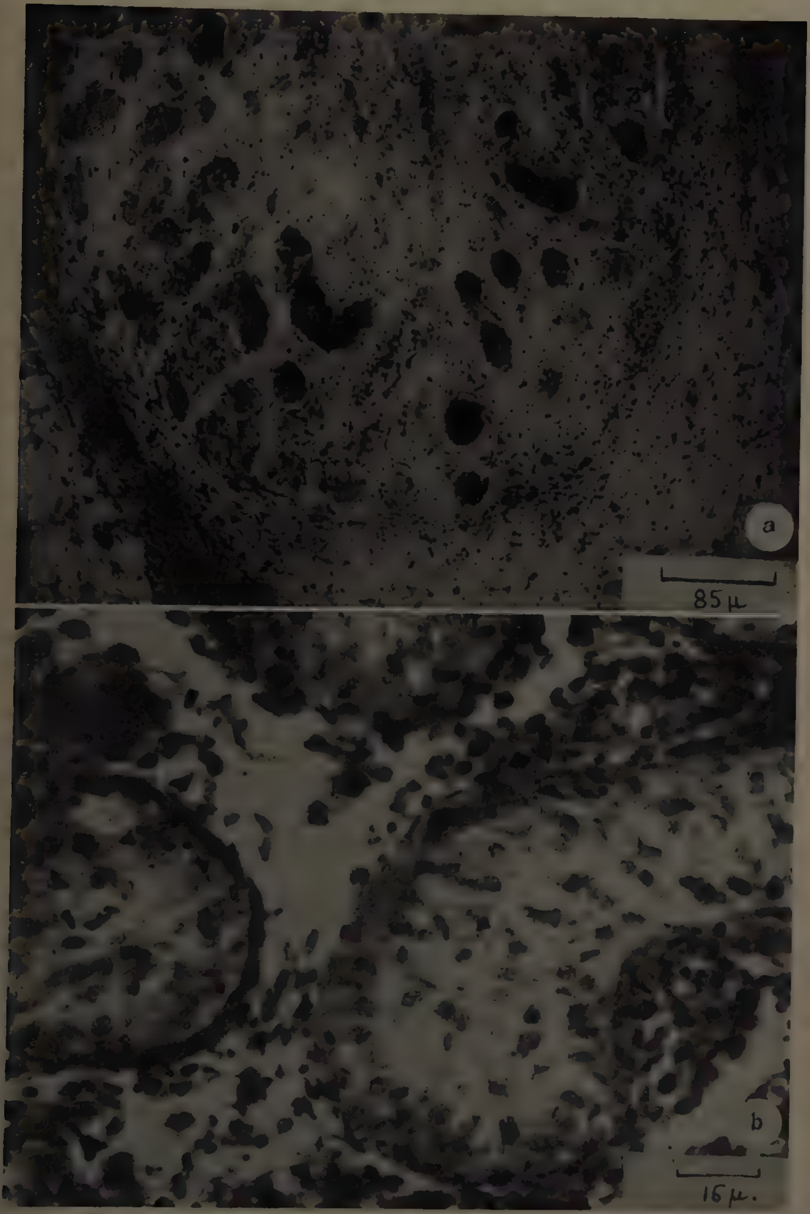

FIGURE 6. (*a*) Mouse B XXXI, 5′; testicular graft from a newborn mouse fixed 21 days after its transplantation under the kidney capsule of a male bilaterally castrated adult mouse; lymphocytic infiltration, pyknotic cells in the tubuli, small development of the interstitial diastematic cells. (*b*) Mouse B XXXI, 5. Testicular graft from a newborn mouse fixed 21 days after its transplantation under the kidney capsule of a male bilaterally castrated adult mouse. Tubuli with spermatogonial-type cells; rather abundant interstitial cells.

kidney capsule, those that came from adult mice and were transplanted in the retrorenal region of similarly bilaterally castrated adult male mice became completely degraded. FIGURE 7 shows such a case (B XXIX, ad 1.). When the host mouse was sacrified 20 days after the graft, the implanted testicle was seen macroscopically as a mass diminished in volume and yellowish and degraded. Histologically, its entire central region was caseous, while its periphery was fibrous and infiltrated by lymphocytes. Only a very few very peripheral tubuli contained germinative cells, spermatogonia, and spermatocytes, some with mitoses, and some Sertoli's cells. Only a very few diastematic cells appeared active.

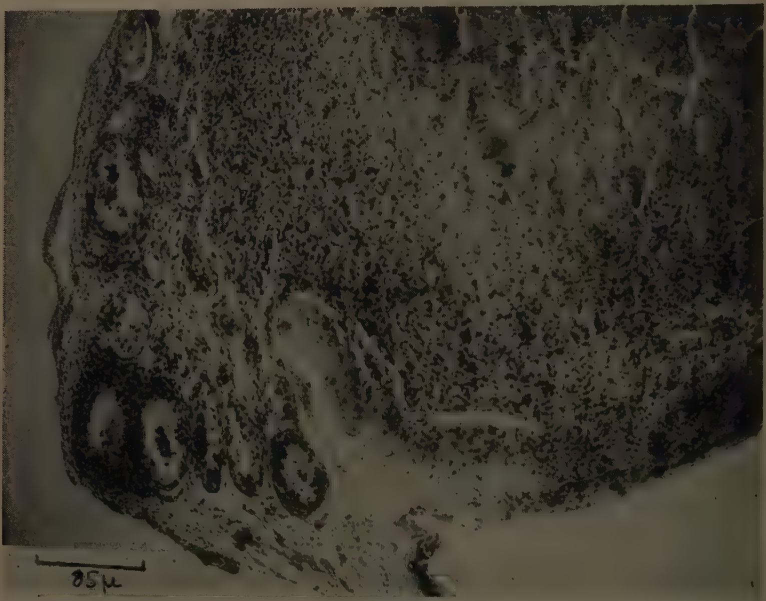

FIGURE 7. Mouse B XXXI, ad. 1. Testicular graft from an adult mouse fixed 20 days after its transplantation in the retrorenal region of a male bilaterally castrated adult mouse. Central region caseous, peripheral region fibrous and infiltrated with lymphocytes. There persist only a very few tubuli on the extreme periphery, with rare interstitial diastematic cells.

The other 3 cases of adult transplanted testicles gave, 36 days after grafting, very similar results.

Thus there is a decreasing series in the development and growth of testicles placed for several weeks under the kidney capsule of male bilaterally castrated mice. The testicles from hypophysectomized mice show a marked enlargement of their tubuli as compared to the contralateral control testicles, a greater development of the germinative cells up to the spermatocyte stage, beyond which they seem unable to go in this site and finally, an enormous development of the interstitial diastematic cells. In the testicles from newborn mice the development of the germinative elements stops at the spermatogonial stage, with a restricted development of the interstitial tissue. Testicles implanted in the adult state become almost totally degraded.

Further control experimentation was found necessary to determine whether testicles from mice hypophysectomized in the infantile state could be induced to develop under the influence of gonadotropic hormone injected (0.1 rat U.) intraperitoneally on 3 consecutive days. Eight such experiments were made, the contralateral testicles excised before the injections serving as controls.

The results have all been comparable. Macroscopically, the 2 testicles—the right (control), and the left, which had been exposed to the injected gonadotropic hormone—are quite similar in appearance. Histologically, the latter occasionally shows tubuli of a greater diameter, up to 80 μ, than those of the control testicle (about 50 μ). This enlargement, when it occurs, is seen especially in the lumen. Sometimes also there are more spermatocytes in these tubuli, and the interstitial tissue is somewhat more developed in the left testicle, which had been exposed to the gonadotropic hormone. In general, however, the 2 testicles show little difference, the left one being quite similar to the right (control) one and resembling those shown in our FIGURES 1, 2 (*right*), and 4 (*right*). It is true that the mice had received only 3 daily injections of gonadotropic hormone, but it is difficult to give more to these fragile hypophysectomized mice, further shocked by a unilateral castration, without provoking a fatal result.

Discussion and Interpretation of Results

The results show very clearly this fact: a testicle from a hypophysectomized mouse that has remained atrophic and inactive during several months nevertheless has potentialities that may be awakened if it is transplanted into a suitable site in an adult mouse, whose gonadotropic hormones act upon it.

The histological examination of testicles from newborn mice shows that, apart from the infantile tubuli, there already exists a relatively important interstitial tissue. While it is inhibited in its evolution by hypophysectomy done during the infantile state, this interstitial tissue nevertheless is apt to develop considerably when it is ultimately exposed to the hormonal action of an adequate host; it then forms characteristic diastematic cells.

It is curious to observe that such a testicle from a hypophysectomized mouse develops relatively better after its transplantation than does a transplanted newborn testicle, in respect of the germinative elements, especially the interstitial tissue. It therefore must be concluded that such inhibited testicular cells, which have only begun their growth in the infantile state, still can be transplanted homoplastically without awakening strong immunological reactions from the host. This state of affairs is similar to that found with transplanted newborn testicular cells, but is quite different from that seen in the transplantation of adult testicular cells. The inhibited testicular cells thus seem to be in a state of slackened vitality that may be compared to the dormant state of vegetable buds and of tooth germs before their unfolding. Such a dormant state enjoys a certain stability, since the testicular elements do not abandon it under the effect of weak doses of gonadotropic hormone, but it yields under the total effect of the hypophyseal hormones of a second organism; the various testicular elements then follow their developmental course.

The dormant state thus seems comparable to, but not identical with, the embryonic state. Like embryonic tissues, dormant testicular tissues show little antigenicity, and therefore can be transplanted to an adult individual of the same species without calling forth reactions that are habitual in homografts, such as are seen in the transplantation of an adult testicle. However, one factor differentiates the dormant from the embryonic state: this is its duration, more variable in the former than in the latter. The normal embryonic state is limited in duration and leads to the adult state in a period of time that is more or less constant for each species. The dormant state, on the other hand, although intimately bound with the embryonic state, nevertheless may be lasting. It is only when dormant tissues, which may remain dormant for a long time, find in their immediate environment the necessary factors for their unfolding and their growth that they resume their evolution toward the adult state.

It remains to be seen whether this difference between the embryonic and the dormant states, which can be proved experimentally in the case of the testicle, can also be shown in other tissues and organs in the course of their development.

Summary

1. Young male albino mice weighing from 8 to 12 gm. were hypophysectomized.
2. Four months later their right testicles were fixed, to serve as controls, and their left testicles were transplanted under the kidney capsule of adult male bilaterally castrated mice.
3. One or two months after their graft the left testicles show tubuli whose diameter is about double that of the tubuli of their right control testicles, and they contain many more spermatogonia and spermatocytes than do the latter.
4. The interstitial diastematic tissue is developed very much more in the grafted testicles than in the contralateral controls.
5. Testicles from newborn mice, transplanted for 1 or 2 months under the kidney capsule of adult male bilaterally castrated mice, take comparatively well, but the development of the germinative cells stops at the spermatogonial stage, and the interstitial tissue shows a limited development.
6. Adult testicles grafted in the retrorenal region of the coelom of adult male bilaterally castrated mice become almost totally degraded.
7. Commercial gonadotropic hormone injected intraperitoneally (0.1 rat U.) on 3 consecutive days into mice hypophysectomized 1 month previously has but little effect on the development of the testicle.
8. The technique of subcapsular transplantation of testicles from mice hypophysectomized before puberty permits the demonstration in these organs of a difference between the embryonic state, which is not durable, and the dormant state, which may be lasting.

Acknowledgment

The technical collaboration of Mireille Hoffmann is gratefully acknowledged.

References

ARON, M. 1953. Transplantation et survie de tissu cancéreux humain dans le testicule du Cobaye. Compt. rend. acad. sci. **237:** 850–851.

ARON, M. 1954. Survie de tissu cancéreux humain transplanté dans le testicule et dans l'épididyme chez le Cobaye. Compt. rend. soc. biol. **148:** 380–383.

ARON, M., A. PETROVIC, C. WEILL & M. DEMINATTI. 1953. Homogreffes dans le testicule du Cobaye. Compt. rend. acad. sci. **237:** 753–754.

ARON, M. 1958. Greffes homoplastiques intratesticulaires chez le Cobaye. Colloques internationaux C.N.R.S. **78:** 25–33.

MAY, R. M. 1932. La transplantation animale. Gauthier-Villars. Paris, France.

MAY, R. M. 1938. Les cellules embryonnaires. N.R.F. Gallimard. Paris, France.

MAY, R. M. 1939. Nouvelles recherches sur la greffe bréphoplastique. Arch. Anat. Microscop. **35:** 147–199.

MAY, R. M. 1952. La greffe. N.R.F. Gallimard. Paris, France.

MAY, R. M. 1954. Nouvelle technique d'hypophysectomie applicable à de jeunes souriceaux. Compt. rend. Assoc. Anat. 40e réunion, Bordeaux. **78:** 255–263.

MAY, R. M. 1955. Induction de la spermatogénèse dans des testicules atrophiés de Souris hypophysectomisées par la greffe bréphoplastique d'une hypophyse sous leur albuginée. Ann. Endocrinol. **16:** 375–382.

PETROVIC, A. 1954. Recherches sur le mode d'action gonadostimulante de la préhypophyse sur le testicule de Cobaye. Thèse de médecine. "Le nouvel Alsacien." Strasbourg, France.

CADAVERIC BLOOD TRANSFUSION

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It is thirty years since the first transfusion of cadaveric blood to a man was performed in the Moscow Sklifosovsky Institute. This transfusion had been preceded by the experiments of Vladimir N. Shamov on dogs, and by his report to the Third Ukrainian Surgical Congress in September 1928. Shamov proved that animals so anemic as to be in the state of clinical death could be revived with the blood of sacrificed dogs. His experiments refuted the belief that cadaveric blood was poisonous. Cadaveric blood is not toxic, and it may be transfused from a dead animal to a live one. It is sterile for several hours, toxicity developing later.

After the death of an animal its blood retains all its qualities. When it has been placed in the circulation system of another organism, the erythrocytes continue to transport oxygen.

When for the first time cadaveric blood was transfused to an engineer who attempted suicide, an excellent clinical result was obtained: the man was saved.

At the end of 1930, Sergei S. Yudin presented to the Fourth Ukrainian Surgical Congress the successful results of transfusing cadaveric citrated blood to seven patients. Two special commissions, military and juridical, stated that Yudin's experiments on cadaveric blood transfusion had a scientific foundation and should be continued.

At that time there were certain practical problems connected with the carrying out of this kind of operation. First, the problem of donors was in the stage of elaboration, usually being solved by volunteers from among a patient's relatives and friends. Second, the present method of blood conservation was then unknown and direct or immediate transfusion of fresh citrated blood was made.

Then it was found that with 5 per cent solution of citrate added, the blood could be kept at temperatures of +3° to +4° C. for two weeks.

In 1932, at the meeting of the National Surgical Society in Paris, France, Yudin reported 100 cases of cadaveric blood transfusion. In France, at that time, the idea of using blood from corpses for transfusion was considered extravagant and unrealistic. Insofar as Yudin's report was concerned, the French surgeons were interested only in the methods of blood conservation because those methods could be used for the conservation of live donor blood.

After 1932, cadaveric blood was accepted for wide clinical application in the U.S.S.R. and, after successful results, scientists began to study the changes occurring in it.

Further extensive investigations made by Maria G. Skundina and Arsenii Russakov proved that this blood had its peculiarities, such as the phenomenon of fibrinolysis. The blood of people after sudden death—without long agony—flows out of the vessels in the liquid state and remains liquid for 20 to 30 min.; then it coagulates but, one-half to one-and-one-half hours later, it becomes liquid again. This process is spontaneous.

The phenomenon of fibrinolysis, that is, the transformation from clot to liquid, is characteristic only of the blood of healthy persons after sudden death. Furthermore, the younger and more healthy the person, the quicker the phenomenon takes place. In cases of disease accompanied by long agony, infection, or trauma with agony, the phenomenon is absent; the blood in such cases coagulates and the clots remain constant until they decay.

Such a property of cadaveric blood was extremely valuable in that it permitted the solution of two important practical questions: how blood might be used (1) without anticoagulants and (2) without conserving preparations. From 1932 on, cadaveric blood was prepared without citrate, that is, as an absolutely unaltered, natural substance without any admixtures.

Biochemical investigations have proved that fibrinemia is not a sign of the disintegration of blood. The phenomenon of fibrinolysis is not accompanied by the dissociation of the protein molecule. Some data show that there is a transformation of the fibrinogen into a morphologically new protein substance.

Thus the phenomenon of fibrinolysis is not only not a sign of destruction and death, but is also a sign of life. Menstrual blood or blood in the abdominal cavity almost always fails to coagulate: here it may be seen that in the live organism some processes similar to fibrinolysis do take place.

In the Sklifosovsky Institute we have a special operation hall for the corpses of persons who have died suddenly. This hall is staffed by a doctor and two nurses 24 hours a day.

The blood of victims of sudden cardiac arrest (myocardial infarction, hypertensive heart disease, alcohol intoxication, and electric shock) is most suitable for use. In cases of sudden death by accident the corpses cannot be used: the crushing of tissues is accompanied by massive hemorrhage, and the danger of infection is present.

Corpses without severe head or brain injuries (not penetrating) may be used.

The corpse affixed to the table is placed in the extreme Trendelenburg position. The left jugular vein (FIGURES 1 and 2), is cut and two cannulas are inserted into it one into the distal end and the second (curved) into the proximal one (FIGURES 3 and 4). The cannulas are fixed in the vein with ligatures and connected to the rubber tubes through which the blood flows (by gravity) into the special sterile ampules (FIGURE 5). Blood in the amount of 2 to 4 liters is usually obtained from each adult corpse (FIGURE 5).

As stated above, cadaveric blood does not need stabilizing agents, but for a longer conservation of 25 to 30 days a glucose-phosphate solution with antibiotics is added, 25 cc. of the solution to 250 cc. of blood. When the maximal amount of blood has been taken, a second portion of the so-called diluted blood is drawn. For this purpose the table is adjusted to the horizontal position and 1 liter of saccharose-glucose solution is injected into the carotid artery. The table is then adjusted to the Trendelenburg position, and about 2 liters of diluted blood is taken.

A lower concentration of erythrocytes and hemoglobin (50 to 60 per cent) is characteristic of the diluted blood. After this procedure the blood is kept in the refrigerator until all laboratory tests have been completed.

The blood group is determined. Bacteriological analysis (growth during 5 days) is made. Malaria, hemoglobin, bilirubin, and cholesterin levels are determined. In some cases concealed hemolysis and erythrocyte resistance are found.

At necropsy a careful macroscopic examination of the organs is made. Only when favorable analytical results are obtained is the blood used in the clinic.

In the Moscow Sklifosovsky Institute we have prepared and transfused cadaveric blood for the last 30 years. More than 30,000 transfusions have been made during this period; the total weight of all the blood used is thus about 30 tons. Cadaveric blood constitutes about 80 per cent of all the blood transfusions made in the Sklifosovsky Institute. Naturally, we use live donor blood as well, chiefly because of blood group compatibility problems.

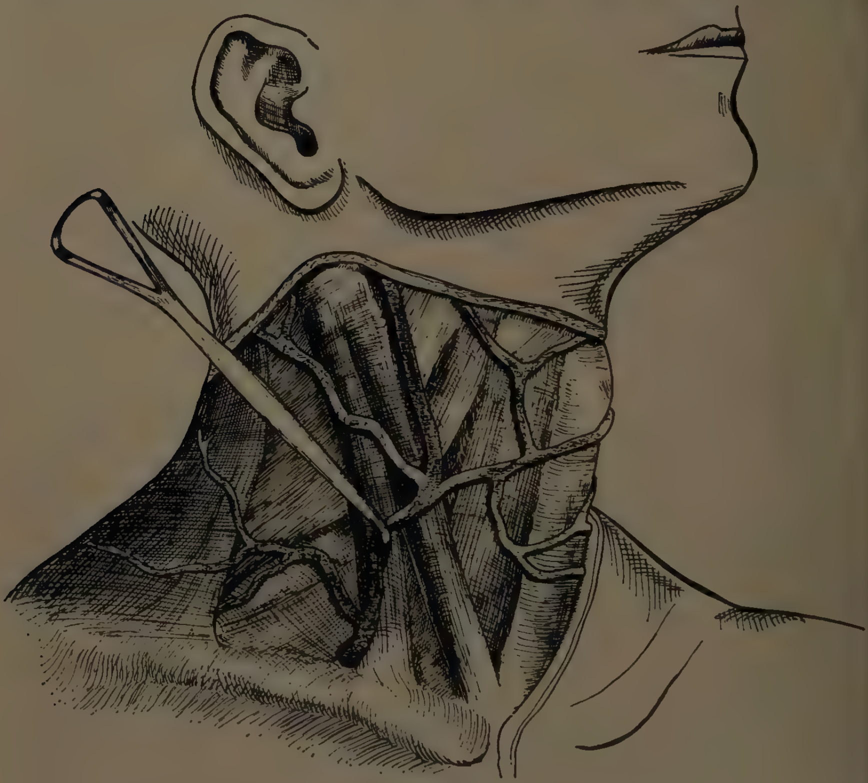

FIGURE 1.

The experience of many years has proved that the use of cadaveric blood represents no danger where infection or toxicity is concerned. Moreover, it has certain advantages over live donor blood.

Discussion of the Paper

WILLIAM DAMESHEK (*Tufts University Medical School, Boston, Mass.*): I think our Soviet colleagues are to be congratulated in this bold escape from the ignorance and superstition of many years' standing regarding the use of cadaveric blood.

Undoubtedly, it helped a great deal during the last world war when the Soviet transfusion institutes found it necessary to cope with the casualties of the German invasion. I was interested in the use of the term "transplantation" in the conference program—namely, "transfusion transplantation," a rather interesting term. Of course, this is a form of transplantation. Every blood transfusion is a form of homotransplantation, but a transplantation in which the great bulk of the cells is composed of more or less dead cells—living, to be sure, but without nuclei and, therefore, without the transplantation antigens that we are accustomed to find in nucleic cellular material.

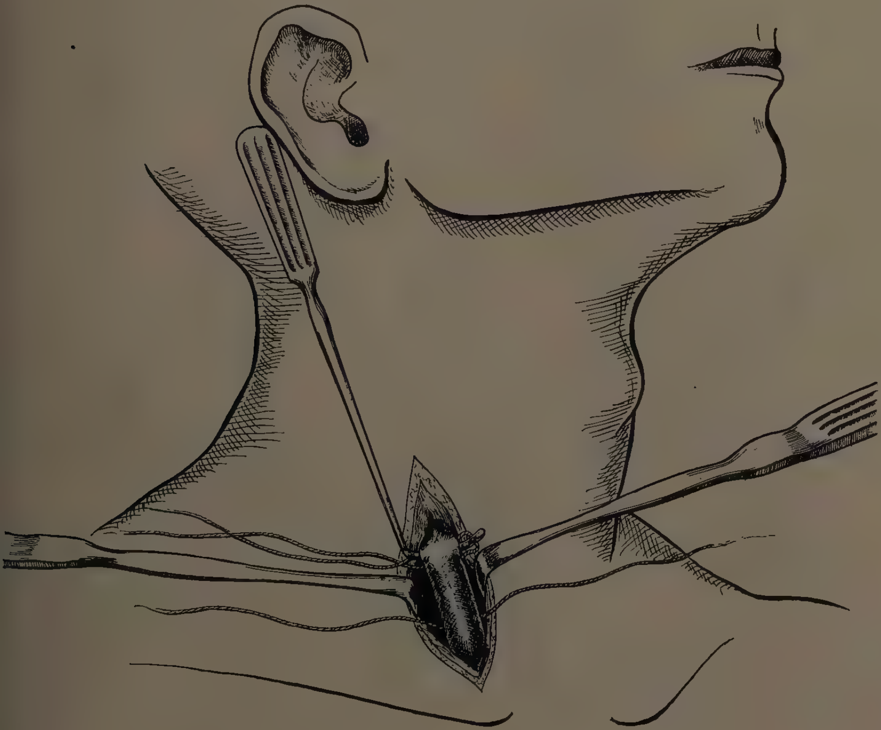

FIGURE 2.

The advantages of the use of cadaver blood are, of course, obvious—during disasters, certainly, for example, during war when a number of fresh young cadavers are available. Moreover, significantly enough, it is possible to collect large quantities of blood from a single cadaver donor.

I note in the conference abstract that one advantage cited by Tarasov is that this blood can be obtained free of charge which, of course, is an advantage. As a side light, however, in the Soviet countries that I have visited, and also the socialist countries that I have visited (not only the Union of Soviet Socialist Republics but Poland, Czechoslovakia, and Hungary), donors do not give blood voluntarily; that is, they do not give blood free of charge. They are paid, and paid a goodly amount according to the currency there, for each donation. The socialist countries make a big point of this, apparently because, I suppose, voluntary giving is a form of charity and, perhaps, the philosophical connotations of charity are not desirable in these Marxist countries.

At any rate, donors in the Soviet Union and in other socialist countries do get paid for their particular blood, which is somewhat of a contrast with our capitalistic economy, where many donors give their blood voluntarily.

With respect to some of the advantages cited, such as the lack of necessity for using citrate and the possibility that reactions may ensue with citrate, it is true that reactions may ensue, but only when very large quantities of citrated blood are given: for example, in an exchange transfusion, but that is not too much of a disadvantage.

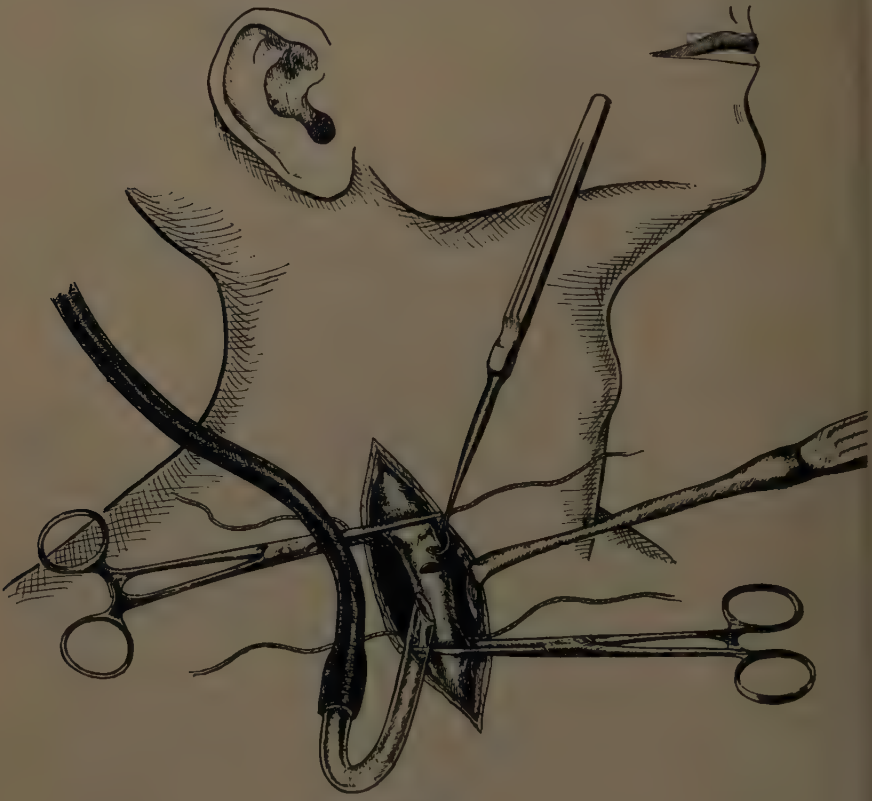

FIGURE 3.

As for post-mortem findings: ruling out disease that you cannot see in the live individual donor, that too, I suppose, is an advantage. I can also see the advantages of detecting any possible bacterial sepsis by post-mortem findings.

I think we can say directly that cadaver blood is valuable for its red cell component, but certainly not for its white cell or for its platelet component because these disappear similarly to those in bank blood; for a hematologist, therefore, this type of blood is not too valuable. We like to use fresh blood containing white cell constituents and platelet constituents. Certainly, the coagulation constituents disappear quickly and the fibrinolytic mechanism is disturbed, in cadaver blood, as has been pointed out, so that cadaver blood is useful only for its red cell component.

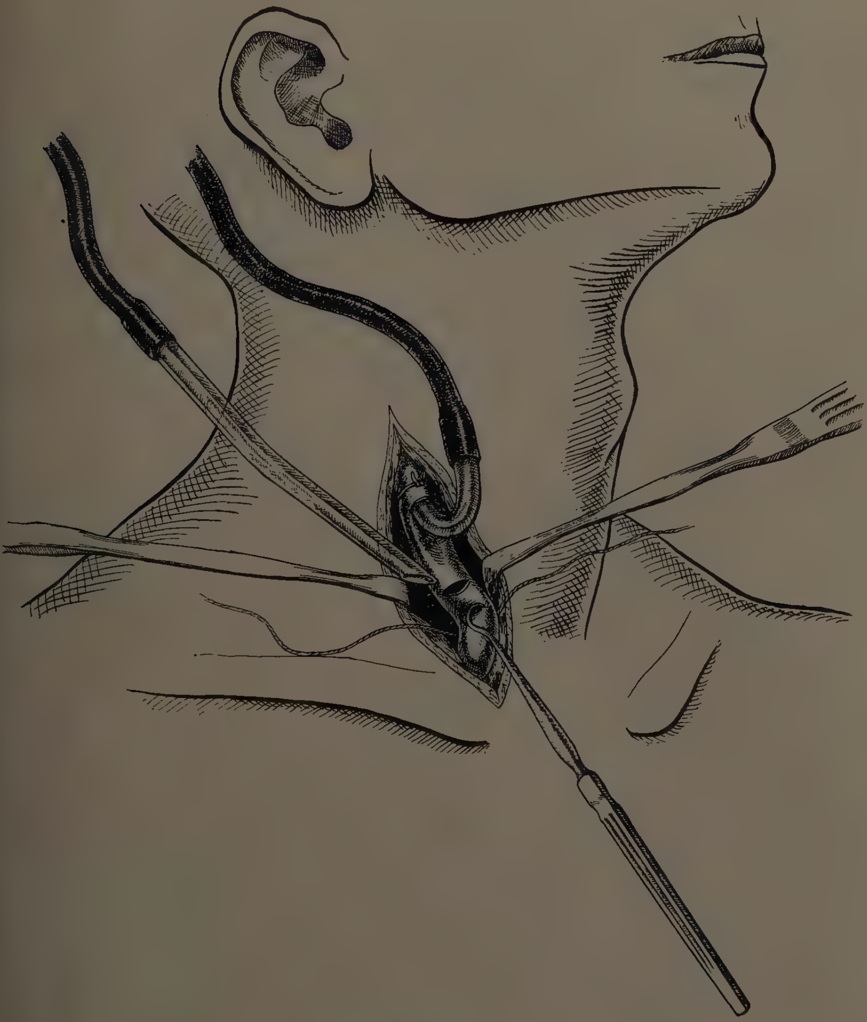

FIGURE 4.

In the United States the usual practice of transfusion as we see it in the clinics under the volunteer system seems to be functioning at the moment rather well; it has not been found necessary to give cadaver blood, with its ancillary problem of obtaining permits from relatives.

I think, however, it should certainly be considered as of possible value in emergencies. It is certainly used a great deal in the Sklifosovsky Institute in Moscow, where large numbers of people are operated upon and where large numbers of cadavers are brought in by ambulance under the central collecting fashion from outlying areas of the city. However, I have observed one curious thing in discussing this question of the use of cadaver blood with various people at other transfusion institutes that I have visited in the Soviet Union and in other countries: they seem to deprecate the use of cadaver blood and

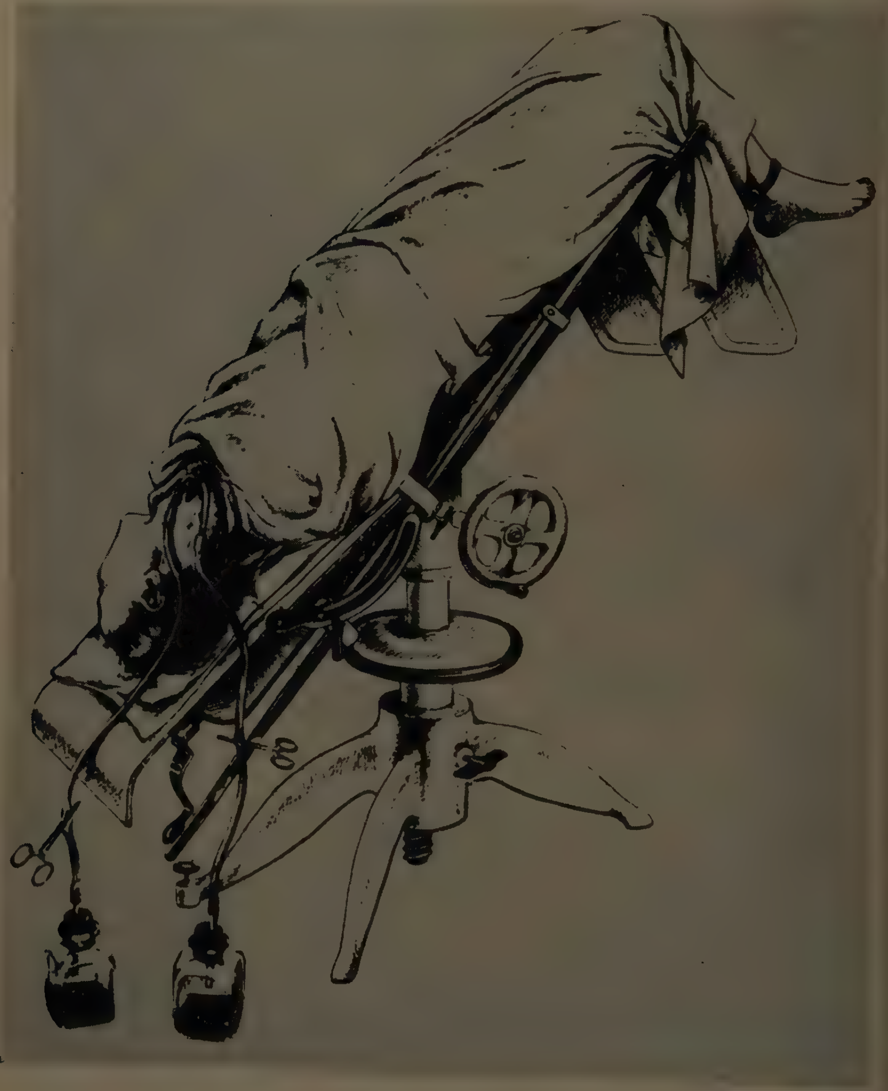

FIGURE 5.

have said it was available or useful only in emergency and first-aid conditions. Thus the practice seems to be somewhat controversial, shall we say, even in the Soviet Union.

However, by way of summary, it must be said that the United States is certainly a wasteful country. There is no question about it. All kinds of things are thrown away. When Europeans and Orientals see us discard syringes, needles, bottles, bags, and things of that sort they are horrified. Moreover, we throw away blood; perhaps it's a bad thing to throw away blood with the deceased body, or to throw away the baby with the bath, to paraphrase an old saying.

It might be a good thing for physicians in the United States to learn the techniques of this transfusion method because, for all we know, disaster may occur; it is advisable, therefore, to be prepared for any emergency.

JOHN C. PRUITT (*Departments of Surgery and Pathology, Bowman Gray School of Medicine, Winston-Salem, N. C.*): Mikhail M. Tarasov should be congratulated for his presentation of valuable information of immediate clinical importance to all of us. I am sure William Dameshek is correct in stating that in some areas of the United States there is no shortage of living blood donors. Unfortunately, however, this is not universally true. For example, in North Carolina there are four institutions in which cardiac surgery is performed, and not infrequently a shortage of blood postpones the proposed procedures. I am sure that most surgeons will agree that every possible source of blood should be explored and fully evaluated, and that the knowledge gained should be widely distributed among physicians. This information, in case of a national emergency, could save thousands of lives. Until Tarasov's presentation today, very few surgeons understood that post-mortem blood was nontoxic.

Cadaver blood has not been used in this country in humans to my knowledge, but the original cadaver blood experiments of Vladimir Shamov have been confirmed here by J. C. Pruitt, J. H. Shinnaberger, I. H. Slade, L. T. Brannon, and T. H. Greer. Beginning in 1954, physical, chemical, and bacteriological studies were performed in our laboratory on blood samples taken from dogs before death and at hourly intervals following death. These studies included direct smear, erythrocyte count, leukocyte count, hematocrit, hemoglobin concentration, hemolytic increment, methemoglobin concentration, serum electrophoretic patterns, and cultures of jugular and portal blood. In addition, one half of the calculated blood volume of other dogs was rapidly removed, precipitating profound clinical shock. These dogs were then transfused with blood removed from cadaver dogs six hours post-mortem five days previously. The electrophoretic pattern studies of cadaver blood had not been previously reported. Our results generally confirm the reports of Shamov and are as follows:

(1) There are no significant changes at 24.2° C. during the first seven hours post-mortem in the erythrocyte count, leukocyte count, red-cell fragility, methemoglobin concentration, or morphology of the blood cells on smears stained with Wright's stain.

(2) Serum electrophoretic patterns indicate that no gross physical or chemi-

cal changes occur in the plasma proteins during the first seven hours following death. The premortem and post-mortem protein fractions appear qualitatively and quantitatively identical.

(3) Blood from the systemic circulation does not become contaminated with bacteria during the first 6 hours following death when the cadaver is kept at 24.2° C. or colder. The seven-hour post-mortem samples were contaminated repeatedly. Blood in the portal system, however, becomes flooded with bacteria during the first hour following death.

(4) The dogs transfused with cadaver blood recovered from shock immediately and demonstrated no reaction to the blood.

Our conclusions indicate that the original Soviet reports are accurate, that blood withdrawn from dog cadavers within six hours can be stored and transfused into living dogs without undesirable reactions, and that the plasma proteins do not undergo significant change.

Studies of fibrinolysin and eventual human transfusion of cadaver blood will be continued at Bowman Gray School of Medicine by C. L. Moore, J. H. Meredith, and by myself, although the overwhelming amount of cadaver blood already successfully and apparently harmlessly transfused in the Union of Soviet Socialist Republics makes our work seem somewhat small and redundant.

We are fortunate to have had Tarasov teach us today about his thirty-year experience with this clinically important procedure.

COMMENT: May I inquire as to the nature and the reason for use of the solution that is described as a preservative?

ROGERS: To put it simply, Dr. Tarasov believes that if the blood is intended for use within the first three weeks after its procurement nothing need be added to the blood as a preservative, but for blood that may remain in the refrigerator for a longer period (25 to 30 days) preservatives are necessary. As stated in the text of his paper, blood required for these longer periods of conservation has added to it a glucose-phosphate solution in combination with a Soviet antibiotic, which I believe is similar to one of our tetracyclines.

From having read of their work, I believe that another reason Tarasov prefers to add a preservative to the older blood specimens is that the longer the blood remains in the refrigerator, the longer the likelihood of changes that might lead to contamination or infection of the blood.

PHILIP LEVINE (*Ortho Research Foundation, Raritan, N. J.*): No mention was made of the possible incidence of subsequent hepatitis, considering the conditions under which the cadaver blood is collected. It is not feasible, I suppose, to obtain a clinical history such as can be obtained in the United States, at least to exclude donors on the basis of a history of jaundice within a certain given time.

I ask, therefore, whether there are any data at all on the incidence of jaundice following transfusion of the cadaver blood.

ROGERS: According to Tarasov and his co-workers, there are very few cases of jaundice in recipients of cadaver blood. When they do occur, they have usually been attributed to some fault in the technique of the personnel who administer the transfusion—that is, contamination during the actual transfusion procedure. Tarasov emphasizes that the autopsy and bacteriological reports usually negate the use of blood from a donor with a history of, or who died of jaundice.

THE RELATIONSHIP OF SERUM GLOBULINS TO TRANSPLANT REJECTION IN THE DOG STUDIED BY PAPER AND IMMUNOELECTROPHORETIC TECHNIQUES*

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While numerous studies have approached the problem of graft rejection by investigating changes occurring in the graft itself, relatively little attention has been focused on the systemic reactions of the host to the graft. On the premise that in the serum proteins changes might be found that would give information as to the nature of the rejection process, electrophoretic studies have been done serially on the sera of dogs receiving kidney homotransplants. Earlier studies by others have shown that homografts of skin[1] and of cornea[2] have no consistent effect on the serum electrophoretic patterns of the host. Our own experience with dogs also indicates that homografts of skin are usually without effect in altering the electrophoretic pattern of serum. On the other hand, it has been found that, in the course of rejection of kidney homotransplants, changes in the electrophoretic pattern of the serum do occur, the most impressive of which is a rise in the level of serum alpha$_2$ globulin.

The first part of this paper describes these changes as they occur with organ homografts and the results of ancillary experiments designed to control the factors of surgery, inflammation, and necrosis associated with organ transplantation and homotransplant rejection. The second part of the study concerns the identification by means of immunoelectrophoretic techniques of the alpha$_2$ globulin involved and its isolation in relatively pure form by fractionation with cold ethanol, followed by column chromatography.

Changes in Electrophoretic Fractions of Serum Proteins in Animals with Homotransplants and in Controls

Methods

The animals used for the study were healthy mongrel dogs. Eight animals with first-set and 3 with second-set homotransplants of kidney were studied. One had a second-set jejunal transplant, and 5 were subjected to autotransplantation of a kidney. The kidney auto- and homotransplants were anastomosed to either the external iliac vessels, the femoral vessels, or the splenic vessels after splenectomy.[3] The site of anastomosis did not appear to influence the results. The jejunal transplant was anastomosed to femoral vessels

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and exteriorized for observation of changes during rejection.[4] Animals receiving second-set grafts had been sensitized to the donor of the graft within the preceding 7 to 35 days by either kidney transplantation or cross perfusion.

Unilateral ligation of a ureter was performed on 3 animals by the extraperitoneal approach, with ligation in the middle third. Retrograde injection of bacteria was combined with this maneuver in 2 experiments in which the effects of pyelonephritis were studied. A third animal in the group with pyelonephritis developed the infection after autotransplantation.

Sterile abscess was produced in 2 animals, in 1 by the injection of Freund's adjuvant and, in the other, by injection of turpentine. Freund's adjuvant was prepared as the usual 8.5:1.5:10 mixture and contained 0.25 mg. of killed tubercle bacilli and 2 mg. of human gamma globulin per milliliter of final mixture. It was injected subcutaneously, 2 ml. at a site. Turpentine was injected subcutaneously after passage through a Seitz filter.

Blood specimens were admixed with a small amount of heparin, and the separated plasma was allowed to clot by the addition of thrombin. Appropriate corrections were applied for the dilution so entailed. This procedure for removing fibrinogen was necessary because whole canine blood, when allowed to clot, frequently yields serum containing hemoglobin that distorts the electrophoretic pattern.

Paper electrophoresis was performed in Veronal buffer at *p*H 8.6, ionic strength 0.075, in a Durrum-type cell. The papers were stained with bromphenol blue dissolved in methanol, and density readings of the stained strips were made in a Spinco Analytrol with a 500 mμ interference filter and a B-5 cam.

Results

Changes in electrophoretic patterns in animals with organ homotransplants. Electrophoresis of serial specimens of serum from the hosts of renal homotransplants showed alterations from the normal pattern in the level of albumin, the alpha$_2$, beta, and gamma globulins. Alpha$_1$ globulin invariably remained constant. The levels of the gamma and, less often, of the beta globulins became elevated in some animals 10 to 12 days after removal of the homograft. The increase in gamma globulin at times amounted to twice its initial value. These changes in gamma and beta globulins, observed a considerable time after graft rejection, were not wholly consistent. Of 5 animals receiving first-set kidney homotransplants in which electrophoretic patterns were followed for prolonged periods, 4 showed the late increase in gamma and beta globulins. One animal receiving a second-set kidney and studied under the same circumstances also showed this effect. Although of interest, these changes have not been investigated thoroughly and will not be dealt with in this paper.

The most consistent change observed was an increase in the alpha$_2$ globulins and a fall in albumin. The pattern almost invariably seen is illustrated in FIGURE 1. In this animal (dog 52) the kidney was anastomosed to the femoral vessels in the groin. In the 8 days after anastomosis urine flow gradually diminished and finally ceased. During this time the level of alpha$_2$ globulin rose slowly from 0.6 gm. per cent to nearly 1.0 gm. per cent. Expressed in terms of percentage of total protein (T.P.), the increase was from 10 to 17 per cent. After the removal of the transplant the level continued to rise, reaching 1.4 gm. per cent (26 per cent of T.P.) 3 days later; thereafter it gradually fell to a normal level in 20 days. Changes in the serum albumin were almost the inverse of those of the alpha$_2$ globulin. Serum albumin fell irregularly from its initial value of 3.7 gm. per cent (56 per cent of T.P.) to a low level of 1.8 gm. per cent (34 per cent of T.P.) at the same time that the alpha$_2$ level was reaching a peak. Thereafter, the level of albumin gradually rose as the level of alpha$_2$ fell. The changes in dog No. 76, shown in FIGURE 2, followed the same pattern. In this animal the renal vessels were anastomosed to the splenics. Two days after removal of the transplant the alpha$_2$ level had reached its maximum of 1.1 gm. per cent (19 per cent of T.P.) coincident with the lowest level of albumin. In each of the 8 animals receiving first-set homotransplants of kidney, a rise in alpha$_2$ globulin with an associated reduction in albumin occurred. Allowing the kidney to remain *in situ* in the host after its rejection, as was done in 1 animal, had no effect on this distinctive pattern of change in serum protein.

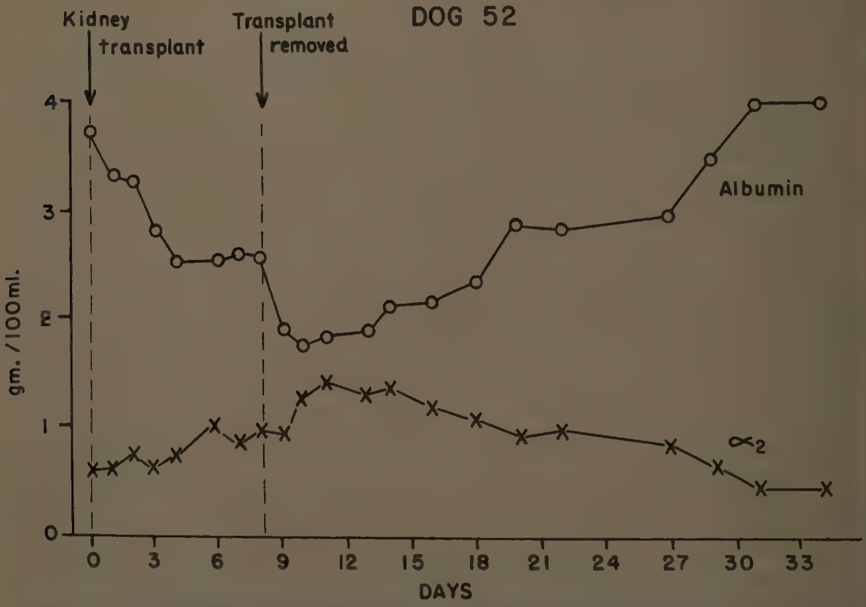

FIGURE 1. Changes in levels of serum albumin and serum alpha$_2$ globulin, measured by paper electrophoresis, in a dog bearing a first-set homotransplant of kidney.

Serial electrophoretic patterns were also obtained in 3 animals receiving second-set kidney homotransplants and in another receiving a second-set jejunal transplant. In 1 of the animals receiving a second-set kidney transplant there was only a slight rise in alpha$_2$ globulin, from 0.6 to 0.9 gm. per cent (9 to 15 per cent of T.P.), although there was a vigorous rejection of the kidney. In the other animals, including the one receiving the jejunal transplant, the electrophoretic changes resembled those seen with the first-set grafts, but occurred over a shorter period. The rapidity of the response is seen for dog 16 in FIGURE 3. In one day the level of $alpha_2$ globulin rose from 0.7 to 1.3 gm. per cent (10 to 22 per cent of T.P.). The continued rise after removal of the kidney again can be seen.

In summary, dogs with first-set kidney transplants and second-set transplants of both kidney and jejunum show an increase in $alpha_2$ globulin and a diminution in serum albumin in the course of the organ rejection. The level of $alpha_2$ continues to rise for several days after the transplanted organ is removed. This continuing elevation can hardly be explained except by continued production of protein after transplant removal, indicating that the protein responsible for the increase in $alpha_2$ globulin does not have its origin in the rejecting homotransplant, but is a response of the host to this insult.

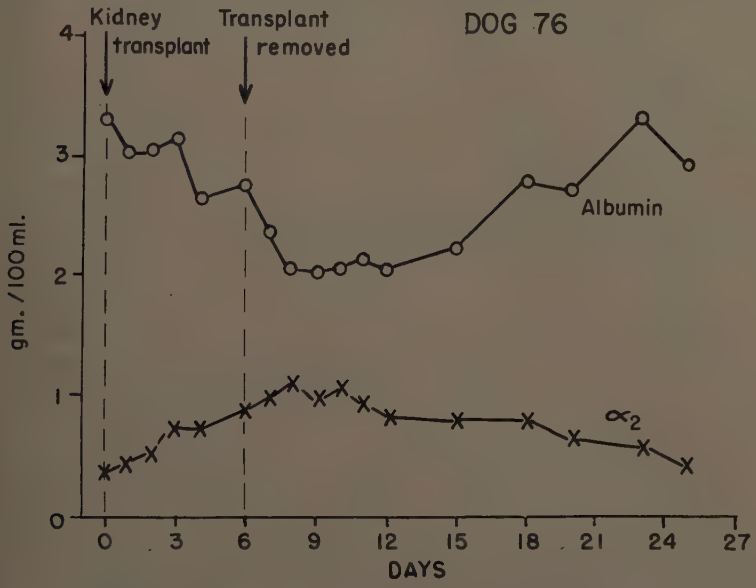

FIGURE 2. Changes in albumin and $alpha_2$ globulin in a dog with a first-set renal transplant.

Effect of renal autotransplantation, renal infarction, and ureteral ligation on albumin and $alpha_2$ globulin. To assess the roles of surgical trauma and of nonspecific renal damage in producing the changes in $alpha_2$ globulin and albumin seen in the animals with homotransplants, studies were done on animals subjected to renal autotransplantation, renal infarction, and unilateral ureteral ligation.

In 5 animals subjected to autotransplantation of a kidney no change was noted in the $alpha_2$ globulin, but there was a decrease in serum albumin similar to that seen in animals receiving homotransplants. Representative experiments are shown in FIGURES 4 and 5. In dog 10 (FIGURE 4) the serum alpha$_2$ actually fell postoperatively after autotransplantation, and there was a moderate fall in serum albumin. In dog 17 (FIGURE 5) the fall in albumin was more marked, resembling that seen in animals with homotransplants. The more profound changes in albumin in this animal were perhaps the result of partial infarction of the kidney, probably occurring shortly after transplantation. A totally infarcted autotransplant in another animal produced a similar change. Despite infarction, the serum alpha$_2$ globulin level did not increase in either animal.

Unilateral ureteral ligation performed in 3 animals caused no increase in serum alpha$_2$ globulin level and no reduction in the level of serum albumin.

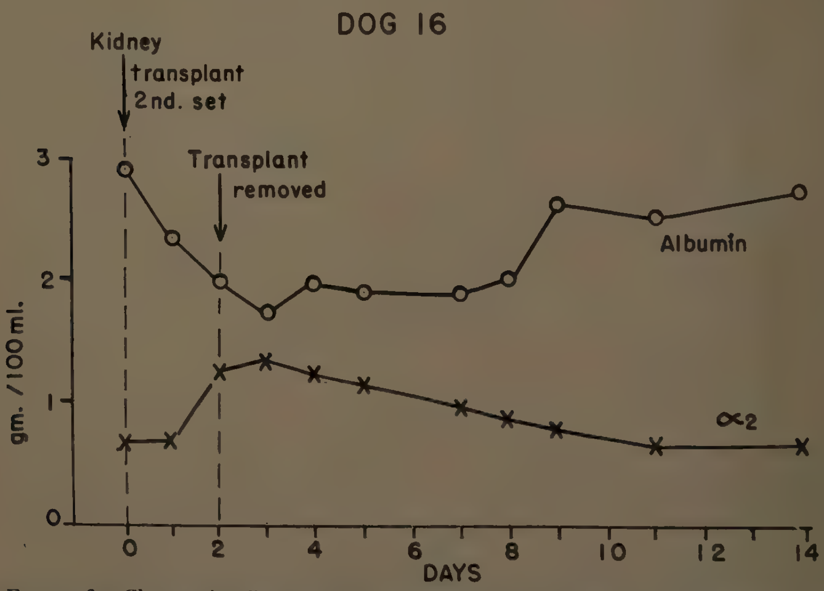

FIGURE 3. Changes in albumin and alpha$_2$ globulin in a dog with a second-set renal homotransplant. The animal was sensitized to the kidney donor by transplantation of a first-set kidney 3 weeks before.

When it is considered that in the present experiments more surgical manipulation was involved in auto- than in homotransplantation, it is obvious that the rise in alpha$_2$ globulin is not the result of surgical trauma. Likewise, tissue destruction accompanied by low-grade inflammation, such as occurs with renal infarction and ureteral ligation, appears to have no effect on the alpha$_2$ globulin level. The data, however, give evidence that surgical trauma and renal infarction do affect the level of serum albumin. Thus it appears that the lowering of albumin is not necessarily accompanied by a rise in alpha$_2$ globulin; it can occur independently as a result of an insult too mild or of the wrong kind to affect the serum alpha$_2$ level.

Changes in electrophoretic patterns produced by experimental pyelonephritis and sterile abscess. It would seem unlikely that an increase in the serum alpha$_2$ globulins would be specifically found only in hosts of organ homotransplants. A study by Siebert *et al.*[5] in 1947 focused attention on increases in alpha$_2$ globulin associated with advanced cancer and tuberculosis and indicated that the protein(s) responsible for the elevation contained carbohydrate in the molecule. Since that time numerous studies have related elevations

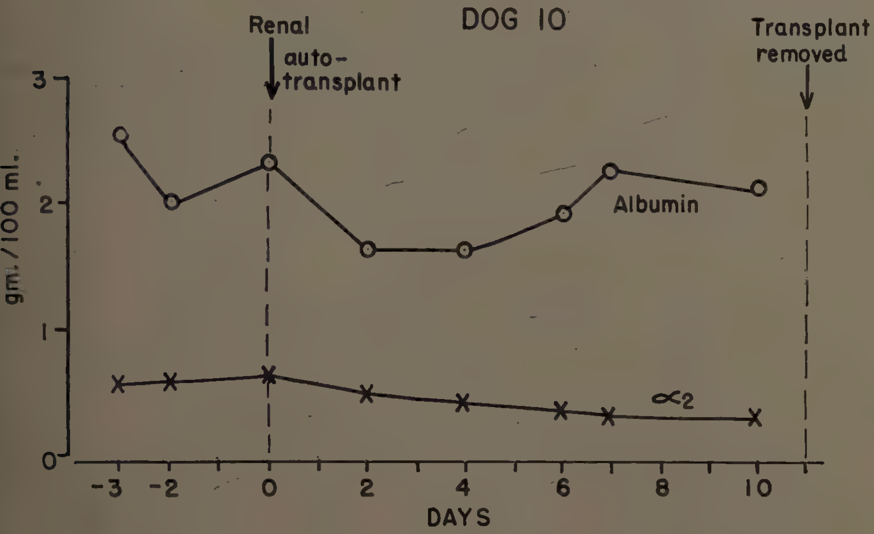

FIGURE 4. Changes in albumin and alpha-2 globulin after renal autotransplantation.

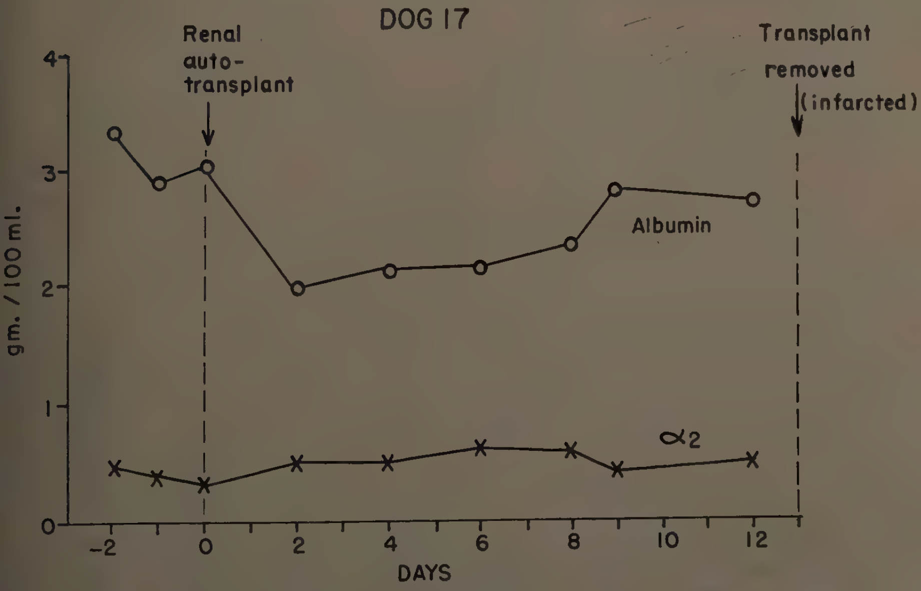

FIGURE 5. Changes in albumin and alpha$_2$ globulin in a dog with an infarcted renal autotransplant.

in serum glycoprotein (measured as glucosamine, nonglucosamine hexose, or seromucoid) to various types of inflammatory reactions. It should be pointed out, however, that the methods used in these studies, whether employing measurement of alpha$_2$ globulin, of carbohydrate, or of seromucoid, are not specific as to the proteins determined. All these methods determine heterogenous mixtures, the components of which have in common only certain electrophoretic mobilities, carbohydrate groups, or specific solubilities in dilute acid. It thus seemed desirable to pursue further the investigation of the rise in alpha$_2$ globulin with renal homotransplantation, first to determine what other conditions cause serum alpha$_2$ globulin levels to rise in the dog and, second, to determine, if possible, whether in these conditions the same alpha$_2$ protein is responsible for the increase in the plasma level or whether different proteins are involved.

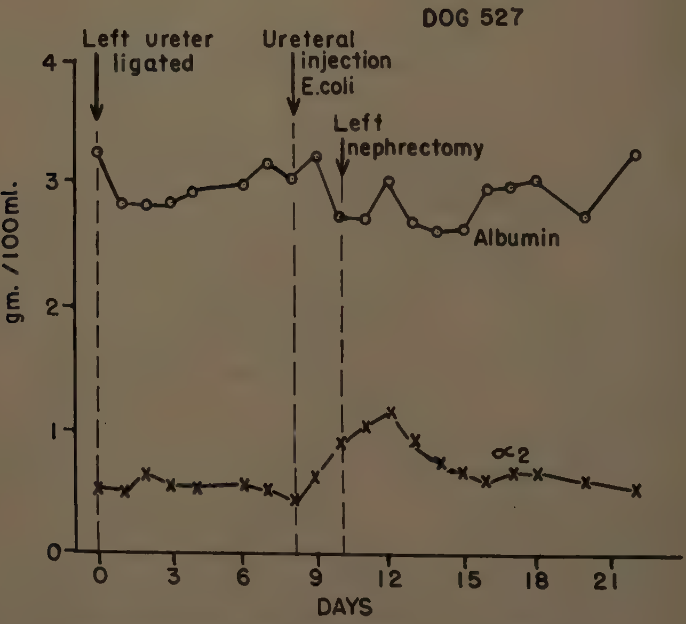

FIGURE 6. Changes in albumin and alpha$_2$ globulin in a dog with experimental unilatera pyelonephritis produced by injection of a suspension of *E. coli* into the stump of the ligated ureter.

In a organ homotransplant undergoing rejection an inflammatory reaction with infiltration of round cells occurs and, at the same time, the transplanted organ undergoes necrosis. To assess the specificity of the rise of alpha$_2$ globulin for transplant rejection, other conditions in which inflammation and necrosis are present have been produced experimentally and the serum patterns followed. The conditions employed were unilateral pyelonephritis and formation of sterile abscess by injection of either turpentine or Freund's adjuvant.

The results in an animal with an experimental pyelonephritis are shown in FIGURE 6. Eight days after ligation of the left ureter a suspension of *Escherichia coli* was injected retrograde into the proximal ureteral stump and, 48 hours later, the left kidney was removed. Fever to 104° F. was present in the period between the injection of organisms and nephrectomy, and the kidney grossly and microscopically showed a moderately severe pyelonephritis. The serum alpha$_2$ level, not affected by ureteral ligation, rose sharply after injection of organisms while pyelonephritis was developing and, as in the animals receiving homotransplants, it continued to rise for 2 days after the kidney was removed. In contrast to other conditions, serum albumin remained constant. Two other animals with unilateral pyelonephritis also showed an elevation of alpha$_2$ globulin. In these animals the disease was more fulminant and the kidney more severely involved; the level of alpha$_2$ globulin went as high as 1.75 gm. per cent (26 per cent of T.P.), exceeding the level of serum albumin.

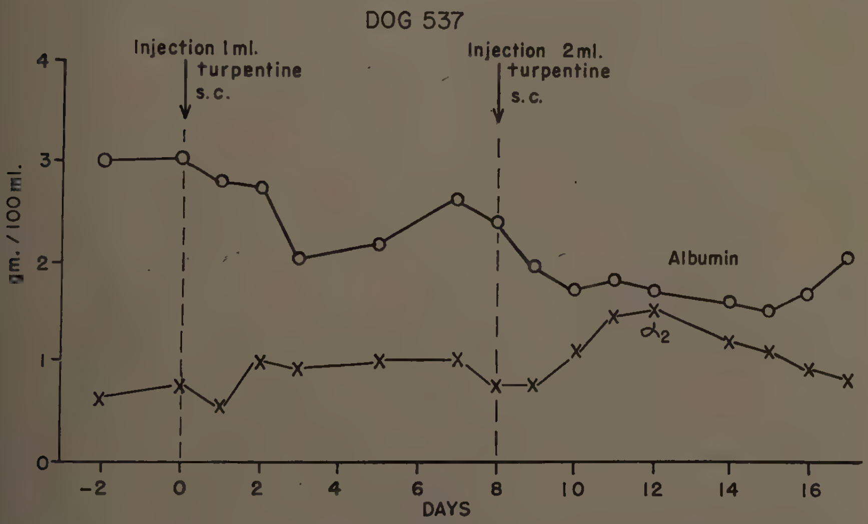

FIGURE 7. Changes in albumin and alpha$_2$ globulin after subcutaneous injection of turpentine.

FIGURE 7 shows the effects of injection of turpentine. After injection of both the 1-ml. and the 2-ml. doses, the alpha$_2$ globulin level remained constant for 24 hours and then began to increase. The time of the rise corresponded to the period when the abscesses were small but increasing in size, and the dog was febrile. The level reached its peak and began to fall before the abscesses reached their maximum size. Levels of serum albumin decreased in a pattern similar to that observed in animals with homotransplants.

An animal receiving subcutaneous injections of Freund's adjuvant on 3 occasions also showed a definite and sustained increase in alpha$_2$ globulin after each injection. The values attained, however, were always less than those seen with turpentine abscess, never exceeding 1 gm. per cent. The lower levels correlated with the fact that the Freund's adjuvant injection did not make the animals so acutely febrile as did the injection of turpentine, and the amount of material spontaneously draining from the abscesses was smaller.

From these studies it is apparent that an inflammatory process, whether resulting from nonspecific renal infection or from injection of either turpentine or Freund's adjuvant, produces the same pattern of change in alpha$_2$ globulin and in albumin as does renal homotransplant rejection.

IDENTIFICATION AND ISOLATION OF THE PROTEIN RESPONSIBLE FOR THE ELEVATION OF ALPHA$_2$ GLOBULIN

Because of the large numbers of proteins of alpha$_2$ mobility in serum, evidence that the responses of dogs with homotransplants and with nonspecific inflammatory conditions are identical can come only through the demonstration that in all these conditions the same protein is responsible for the rise in alpha$_2$ globulin. To this end determinations of seromucoid and of protein-bound hexose were done on sera of animals with homotransplants to determine whether the protein responsible for the alpha$_2$ changes would fall in the glycoprotein class. As reported in the literature for other types of inflammatory conditions, the levels of these substances rose to parallel in general the rise in alpha$_2$ globulin. It was then found by immunoelectrophoretic analysis that sera with a high alpha$_2$ content from animals with homotransplants, when reacted with antisera to dog alpha$_2$ globulins, showed a large and well-defined arc in the alpha$_2$ globulin region, the counterpart of which in normal serum was considerably smaller. It was inferred from these observations that a single protein was responsible in the homotransplant animals for the elevation of alpha$_2$ globulin, and that this protein was a glycoprotein. To pursue the observations further, the method devised by Schmid *et al.*[6] for the isolation of a glycoprotein with alpha$_2$ mobility from Cohn Fraction VI of human plasma was applied to this fraction of dog plasma. By reactions of identity on the immunoelectrophoretic plate it has been demonstrated that this glycoprotein is the one responsible for at least the major portion of the increase in alpha$_2$ globulin, not only in animals with homotransplants, but also in those with nonspecific inflammatory conditions.

Methods

Seromucoid was determined on plasma defibrinated with thrombin by the method described by Weimer and Moshin.[7] Considerable care was taken to add the diluted serum to the perchloric acid under the same conditions in each run. The determination of the phosphotungstic precipitate of seromucoid utilized the Folin-Ciocalteau procedure as modified by Lowry.[8] The method for protein-bound hexose utilized the method of Lustig and Langer[9] as modified by Weimer and Moshin.[7] The results were expressed in terms of a standard consisting of equal parts of galactose and mannose.

The antisera used for immunoelectrophoretic analysis were prepared against the alpha$_2$ fraction of dog serum obtained by continuous-flow paper electrophoresis. Fractionation was performed with a Beckman-Spinco model CP cell kept at 4° C. with a Veronal buffer, *p*H 8.6, ionic strength 0.02. Collecting tubes were changed every 4 hours. Difficulties were not experienced with changes in the course of migration during a run. Tubes containing the alpha$_2$ globulins were selected by referring to the pattern on the curtain and stained after completion of the procedure. The buffer-protein mixture collected was incorporated directly into Freund's adjuvant after adjusting the *p*H to 7.0 and adding NaCl to a concentration of 0.15 *M*. The serum and the products of the curtain fractionation were not stored. Instead, the adjuvant was prepared on the day following an overnight run on the CP cell and injected immediately into the rabbits and goats used for preparation of the antisera. Injections were made on 4 occasions, 7 to 20 days apart. The animals were bled 5 to 7 days after the last injection.

The technique of electrophoresis in agar for immunoelectrophoretic analysis was that described by Grabar.[10]

Fractionation of dog plasma to obtain Fraction VI was performed on samples of plasma varying in volume from 90 to 280 cc. by the procedure described by Cole and Favour.[11] The yield exceeded that obtained from human plasma, averaging about 160 mg./100 ml. of initial plasma volume. The final product was lyophilized and then dissolved in 0.05 *M* citrate buffer, *p*H 5.20, for column chromatographic separation of alpha-2 glycoprotein by the method of Schmid *et al.*[6] The products of the column were dialyzed against several changes of distilled water, lyophilized, and stored at −10° C. for later use for identification procedures.

Results

Changes in serum alpha$_2$ globulin, seromucoid, and protein-bound hexose in a single experiment can be compared in FIGURE 8. Seromucoid levels paralleled almost exactly the changes in alpha$_2$ globulin, but were consistently at a lower level, with seromucoid protein about 40 per cent of alpha$_2$ protein value. Protein-bound hexose showed some tendency to increase in parallel with the alpha$_2$ level, but the changes were much less impressive.

The results of immunoelectrophoretic analysis of sera from a normal dog and from an animal subjected to renal homotransplantation 9 days previously are shown in FIGURE 9. The abnormal serum (66-9) in the upper section had, by paper electrophoresis, an alpha$_2$ globulin level of 1.2 gm. per cent, while the normal serum (NDS) in the lower section had an alpha$_2$ level of 0.5 gm. per cent. The points of origin lie under the labels. Albumin migrated to the right, out of the picture, and the alpha$_2$ globulins are located in the area immediately to the right of the origin. The antiserum used to develop the pattern was obtained from a rabbit immunized with alpha$_2$ globulins obtained by curtain electrophoresis of sera of dogs bearing renal homotransplants. The antiserum contained, by chance, antibodies to only gamma globulin and a single alpha$_2$ globulin. This alpha$_2$ globulin, distinguished in the normal serum as a relatively small arc, is a broader, longer, and heavier arc in the abnormal serum. The difference in size of the arcs leaves little doubt that this globulin was in considerably greater concentration in the abnormal serum.

Similar results were obtained with all sera having a high alpha$_2$ level, whether from animals with organ homotransplants, pyelonephritis, turpentine abscess, or the sterile abscess that follows injection of Freund's adjuvant. Antisera obtained from nearly all rabbits and goats immunized with alpha$_2$

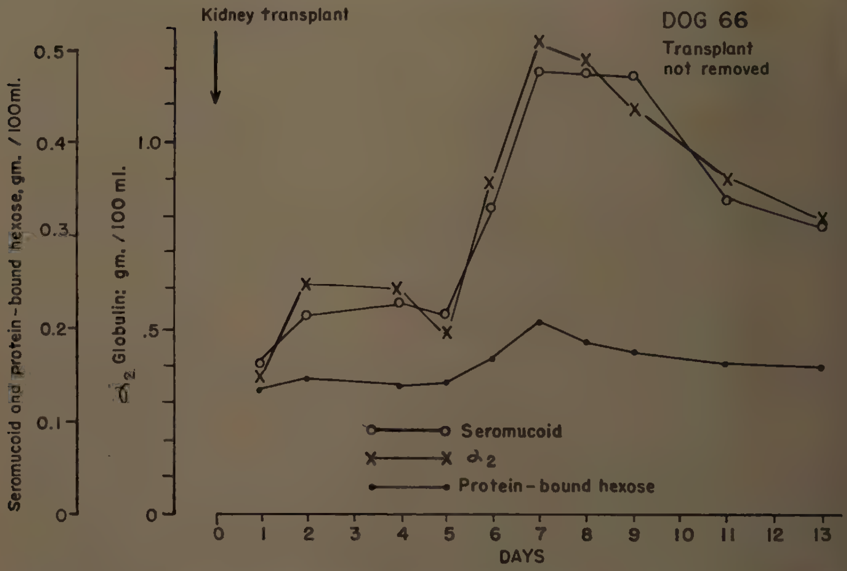

FIGURE 8. Changes in alpha$_2$ globulin, seromucoid protein, and protein-bound hexose following renal transplantation.

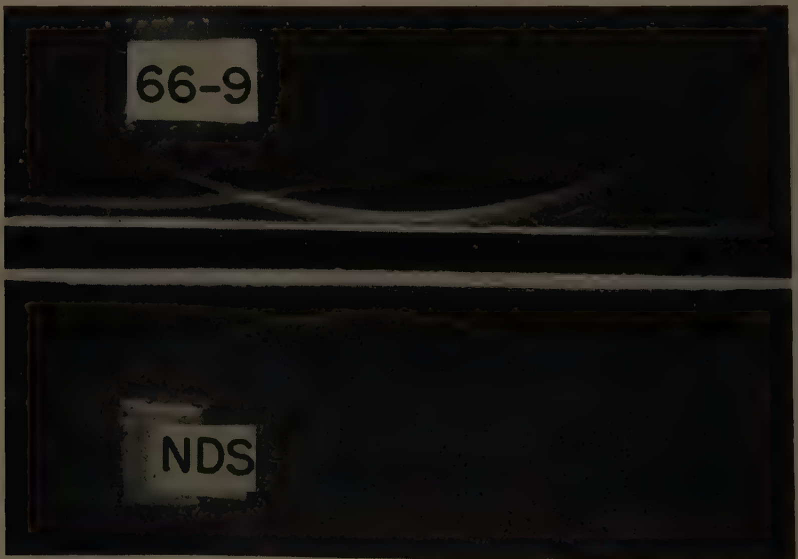

FIGURE 9. Photograph of the alpha$_2$ region of an immunoelectrophoretic plate. Above, electrophoresed dog serum with a high alpha$_2$ level and below, normal dog serum. The trough between contained rabbit antidog alpha$_2$ globulin. The flat precipitin line to the left represents gamma globulin.

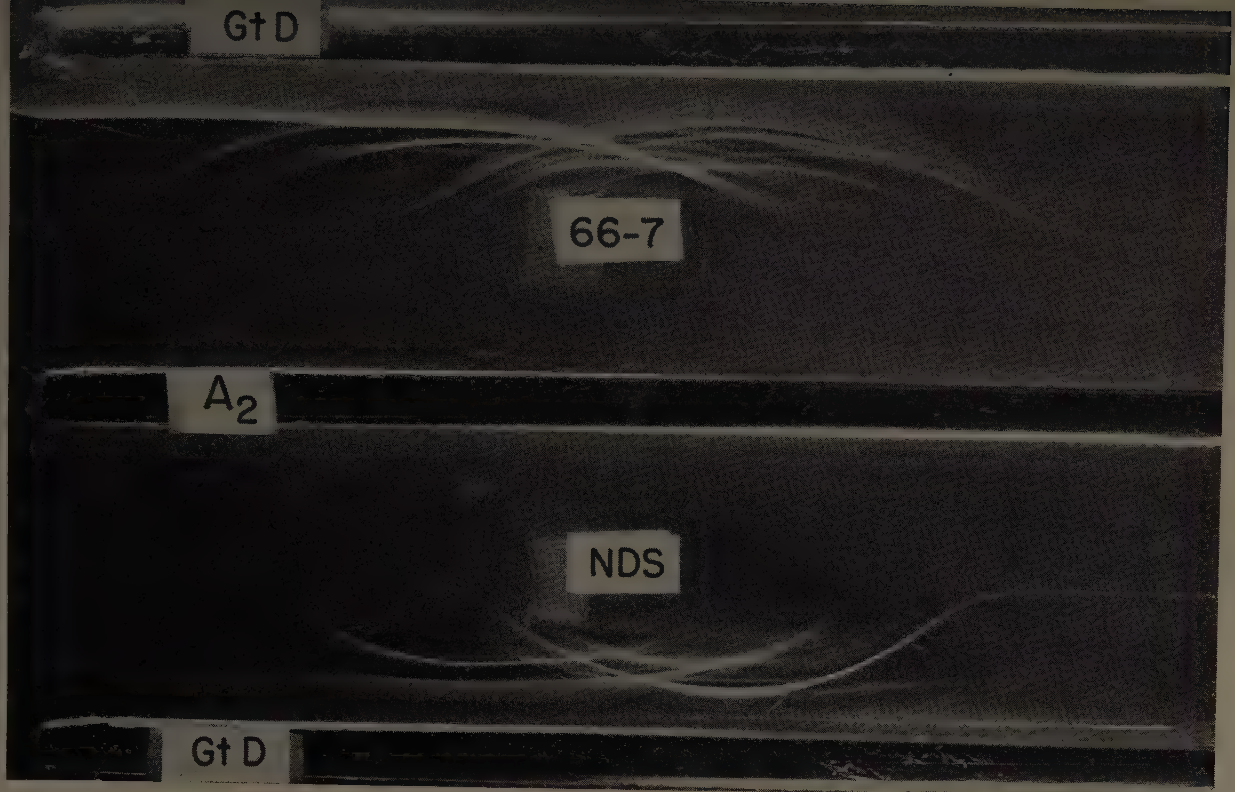

FIGURE 10. Immunoelectrophoresis of abnormal serum (66-7) and normal dog serum (NDS) with development to show reaction of identity between the precipitin arc produced by a serum alpha$_2$ globulin and the precipitin line produced by the alpha$_2$ glycoprotein isolated by column chromatography (A$_2$). The lines representing beta, gamma, and other alpha$_2$ globulins show artifacts caused by antibody excess and by the long incubation necessary for development of the reaction of identity.

globulin were effective in demonstrating this protein. Most of the antisera contained antibodies to a number of proteins in addition to the alpha$_2$ in question. The patterns developed by these antisera containing multiple antibodies indicated that the other alpha$_2$ proteins of normal and abnormal sera were essentially similar. Thus, within the limits of the rough quantitation possible with immunoelectrophoresis, it would appear that the elevation of alpha$_2$ globulin in these experiments is attributable to a single protein, presumably a glycoprotein.

Application of the isolation procedure of Schmid[6] to Fraction VI of dog plasma yielded an alpha$_2$ glycoprotein preparation that was less pure than that obtained by Schmid from human plasma. Paper electrophoresis at *p*H 8.6 showed it to be contaminated to the extent of 26 to 30 per cent with serum albumin. This contaminant does not, however, interfere with the identification of the alpha$_2$ glycoprotein constituting the bulk of the mixture.

To identify the alpha$_2$ glycoprotein, reactions of identity by immunoelectrophoresis as described by Osserman[12] were employed. Abnormal (66-7) and normal (NDS) sera were subjected to electrophoresis in parallel in agar, as shown in FIGURE 10. In a trough cut between them was placed a solution of 5 mg./ml. of the alpha$_2$ glycoprotein obtained from the resin column by the method of Schmid (A$_2$). In troughs on opposite sides of the electrophoresed sera was placed a goat antiserum (Gt D) that contained antibodies to many alpha$_2$ and beta$_1$ proteins, including the alpha$_2$ glycoprotein in question. The antiserum, in addition to developing arcs of precipitate with the sera, eventually reacted with the alpha$_2$ globulin, diffusing in both directions from the middle trough to produce a line of precipitate parallel to the electrophoretic axis. This line will fuse with the ends of the precipitin arc from serum that represents the same antigen-antibody reaction, resulting in a reaction of identity. Such a reaction, as may be seen in FIGURE 10, occurred in this case. The ends of the arcs representing the protein responsible for the elevation of the alpha$_2$ globulin and its counterpart in normal serum have fused with the precipitin line produced by the alpha$_2$ globulin of Schmid. The presence of only single lines parallel to the electrophoretic axes makes it apparent that, if other alpha$_2$ globulins are present as impurities with the alpha$_2$ glycoprotein, they do not have corresponding antibodies in this goat antiserum. It should be noted that the alpha$_2$ globulin arc representing this glycoprotein in the homotransplant serum (66-7) is again much broader than that in the normal, reflecting the abundance of this protein in the animal bearing the homotransplant. In the sera of animals with pyelonephritis and sterile abscess similar reactions of identity occurred between the serum alpha$_2$ globulin in high concentration and the isolated alpha$_2$ glycoprotein.

Discussion

With the evidence that the same protein is responsible for the increased levels of alpha$_2$ globulin in all conditions studied, it becomes apparent that the reaction of the host to an organ transplant, as seen in the albumin-alpha$_2$ globulin portion of the electrophoretic pattern, is a form of acute phase reaction elicited by a number of insults. There is no evidence that this alpha$_2$ protein is related to the formation of transplant antibody by the host; instead, it apparently reflects the inflammatory reaction and perhaps the necrosis that is a part of the rejection process. The absence of these electrophoretic changes with skin and corneal grafts may be attributable to the small mass of these grafts as compared to that of organ grafts, and perhaps also to the fact that the products of the inflammatory reaction drain to the external environment rather than remaining in the host.

Earlier work on the acute-phase reaction accompanying turpentine abscess, a condition that has an effect similar to organ transplant rejection, indicated that at the height of the reaction marked changes in protein metabolism occur. Rates of synthesis and degradation of both albumin and fibrinogen are greatly increased,[13] and hemoglobin production is slowed,[14] while the turnover of globulins, taken as a whole, is little affected.[13] In all probability, the rise in the specific glycoprotein recorded in this paper is linked in some way with these diverse changes.

A number of conditions are known to be associated with increased glycoprotein concentration in the plasma. These were summarized several years ago in a review by Winzler.[15] Included are clinical and experimental neoplasm, tuberculosis pneumonia, rheumatoid arthritis, burns, immunizations and, of the noninflammatory conditions, injection of parathormone and ACTH. The methods of protein fractionation and carbohydrate determination in these studies were not sufficiently specific to determine whether the alpha$_2$ glycoprotein identified in the present study was responsible for the elevation in all these conditions. The study of Siebert *et al.*[5] of serum protein in clinical cancer and tuberculosis, showing parallel changes in alpha$_2$ globulin and serum glycoprotein, suggests that in these conditions the same alpha$_2$ glycoprotein is involved as in the present investigation.

The exact stimulus responsible for the increased glycoprotein cannot be determined from our observations, nor is it known for the glycoprotein response in general. From the present study, inflammation and necrosis are equally eligible as trigger mechanisms, although it is apparent that necrosis and atrophy with only a minimal inflammatory response will not produce a rise in alpha$_2$ globulin, as evidenced by the negative results in the animals with infarcted autotransplants or ligated ureters. It is of interest that an inflammatory reaction composed mainly of cells of the lymphocyte-plasma cell series, as in kidney homotransplants and Freund's abscess, was as effective as turpentine abscess and pyelonephritis (reactions in which polymorphonuclear leukocytes predominate) in calling forth the glycoprotein response. The brisk glycoprotein rise with turpentine abscess indicated that this response can occur in the absence of activity of the immune system. Both tissue destruction[5,16,17] and tissue proliferation[18] have been suggested as common denominators of the glycoprotein response. In this regard it is pertinent to note that the suggestion made by others[15,16,17] that the glycoproteins may arise at the site of inflammation or necrosis is not borne out in the circumstances of the present study, inasmuch as surgical removal of the inflamed necrotic tissue was followed by a further increase in serum glycoprotein level. Equal in obscurity to the trigger mechanism is the purpose of the glycoprotein response and the role it plays in the inflammatory emergency. It could be imagined that the glycoprotein, either directly or indirectly, affords the organism some protection from the insult producing the inflammation. On the other hand, it is equally possible that the response is only a by-product of the gross changes in metabolism occurring with inflammation and has no real purpose.

SUMMARY AND CONCLUSION

In dogs, the rejection of a homotransplant of kidney or jejunum was accompanied by a reduction of serum albumin and an increase in serum alpha$_2$ globulin. The changes were such that the lowest values for serum albumin usually coincided with the peak values for alpha$_2$ globulin. After removal of the organ, the level of albumin usually continued to fall and alpha$_2$ level to rise, indicating that the protein responsible for the latter change does not have its source in the rejecting organ.

An increase in alpha$_2$ globulin also occurred in animals with nonspecific inflammatory conditions such as pyelonephritis and sterile abscess produced by injection of Freund's adjuvant or turpentine. An increase was not seen in animals with autotransplants of kidney, with or without renal infarction, or in animals with unilateral ureteral ligation. The serum albumin, on the other hand, tended to fall both in animals with autotransplants and in those with the nonspecific inflammatory conditions; the changes were not as consistent as those occurring in the animals with renal homotransplants.

By immunoelectrophoretic analysis it was possible to identify by its size and shape a single precipitin arc that represented the protein responsible for the rise in alpha$_2$ globulin. This protein was isolated from Cohn Fraction VI of dog plasma by applying methods used by others to isolate the corresponding protein from human plasma. The protein is a glycoprotein and has been found, by reactions of identity on the immunoelectrophoretic plate, to be the protein responsible for the elevation of alpha$_2$ globulin in all conditions studied.

The similarity of the changes in electrophoretic patterns in animals with renal homotransplants, sterile abscesses, and pyelonephritis, together with the fact that in all these conditions the same protein is responsible for the elevation in alpha$_2$ globulin, puts the response in the class of an acute-phase reaction. Not being specific for homotransplant rejection, the changes cannot be a part of the antigen-antibody reaction responsible for transplant rejection, but seemingly occur only in response to the inflammatory reaction present in the rejected organ. The fact that the level of alpha$_2$ glycoprotein continues to rise after removal of the inflamed organ indicates that this protein does not arise at the inflammatory site, but is produced elsewhere in response to some stimulus. Whether the stimulus is mediated by the inflammatory reaction or by the accompanying tissue necrosis, or both, is not known.

ACKNOWLEDGMENTS

This study has been dependent upon the technical skill and assistance of many individuals, including in particular the contributions of Catherine Golden, Susan Van Ausdal, Norma Myers and June Baker of the Children's Hospital Research Foundation and of Barbara Bouldin of the May Institute for Medical Research of the Jewish Hospital.

REFERENCES

1. WERDER, A. A., C. A. HARDIN & P. MORGAN. 1957. Electrophoretic studies on serum proteins of mice, guinea pigs and rabbits with autologous or homologous skin grafts. Federation Proc. **16**(1): 376.
2. KLEN, R. & M. KÁNSKÝ. 1957. Level of gamma globulin postoperatively after perforating keratoplasties: a preliminary report on 14 cases. Ann. N. Y. Acad. Sci. **64**(5): 929.
3. NATHAN, P., L. J. WILCHINS, H. PESCOVITZ, E. C. FOULKES & B. F. MILLER. 1959. Homologous transplantation of the dog kidney to the spleno-portal vessels. Federation Proc. **18**(1): 292.
4. MACK, J. R., P. NATHAN, B. C. WEXLER, E. GONZALES & B. F. MILLER. Homotransplantation of the dog jejunum. Transplantation Bull. In press.
5. SEIBERT, F. B., M. V. SEIBERT, A. J. ATNO & H. W. CAMPBELL. 1947. Variation in protein and polysaccharide content of sera in chronic diseases, tuberculosis, sarcoidosis, and carcinoma. J. Clin. Invest. **26**: 90.
6. SCHMID, K., M. B. MACNAIR & A. F. BÜRGI. 1958. Chromatographic separation and purification of acidic proteins on carboxylated ion exchange resins. J. Biol. Chem. **230**: 853.
7. WEIMER, H. E. & J. R. MOSHIN. 1953. Serum glycoprotein concentrations in experimental tuberculosis of guinea pigs. Am. Rev. Tuberc. **68**: 594.
8. LOWRY, O. H., N. J. ROSEBROUGH, A. L. FARR & R. J. RANDALL. 1951. Protein measurement with Folin phenol reagent. J. Biol. Chem. **193**: 265.
9. LUSTIG, B. & A. LANGER. 1931. Über die Bestimmung und den Gehalt an freiem Eiweiss—und gebundenem Nichteiweisszucker im normalen und pathologischen Seren, Liquor und Harn. Klin. Wochschr. **10**: 2133.
10. GRABAR, P. 1959. Immunoelectrophoretic analysis. *In* Methods in Biochemical Analysis. **7**: 1. D. Glick, Ed. Interscience. New York, N. Y.
11. COLE, L. R. & C. B. FAVOUR. 1955. Correlations between plasma protein fractions, antibody titers and passive transfer of delayed and immediate cutaneous reactivity to tuberculin PPD and tuberculopolysaccharides. J. Exptl. Med. **101**: 391.
12. OSSERMAN, E. F. 1959. Identification of specific bands in immuno-electrophoresis by concurrent interaction with known protein solutions. Federation Proc. **18**(1): 589.
13. YUILE, C. L., F. V. LUCAS, C. K. JONES, S. J. CHAPIN & G. H. WHIPPLE. 1953. Inflammation and protein metabolism studies of carbon-14-labeled proteins in dogs with sterile abscesses. J. Exptl. Med. **98**: 173.
14. WINTROBE, M. M., G. R. GREENBERG, S. R. HUMPHREYS, H. ASHENBRUCKER, W. WORTH & R. KRAMER. 1947. Anemia of infection. III. Uptake of radioactive iron in iron-deficient and pyridoxine-deficient pigs before and after acute inflammation. J. Clin. Invest. **26**: 103.
15. WINZLER, R. J. 1955. Determination of serum glycoproteins. *In* Methods of Biochemical Analysis. **2**: 279. D. Glick, Ed. Interscience. New York, N. Y.
16. CATCHPOLE, H. R. 1950. Serum and tissue glycoproteins in mice bearing transplantable tumors. Proc. Soc. Exptl. Biol. Med. **75**: 221.
17. ENGEL, M. B. 1952. Mobilization of mucoprotein by parathyroid extract. A.M.A. Arch. Pathol. **53**: 339.
18. SHETLAR, M. R., *et al.* 1949. Serum polysaccharide level in malignancy and in other pathological conditions. Cancer Research. **9**: 515.

DISCUSSION OF THE PAPER

MURRAY: Fisher has carried out some studies on the rejection of kidney transplants by using fluorescent antibodies. Would he say a word on that?

BERNARD FISHER: The only thing I can say is that we have already published these studies using the fluorescent antibody techniques in the *Journal of Negative Information*. We have subsequently made the same studies, using spleen transplants, and we have elaborated upon our investigations a little by studying animals in which we removed the transplanted kidney and then took the serum several days later to try to find circulating antibody, but all the results were still negative.

MERRILL: Many years ago when Simonsen did his work on transplanting kidneys in dogs, he reported finding a hemagglutinin as evidenced by the agglutination of donor cells by the serum of the kidney recipients. However, he also noted a phenomenon of panagglutination whereby the recipient animals, at least in some incidences, as I remember it, agglutinated their own red cells and those of normal donors. At the time, he suggested that one explanation for this phenomenon might be some nonspecific factor arising from tissue necrosis as a result of rejection of the kidney.

I wonder if this phenomenon of increased α_2 globulin, might possibly be the type of thing that Simonsen observed, and I ask whether West performed any red cell agglutinations that might possibly be correlated with the rise in his protein.

NORMAN COOPER: I believe that our lack of knowledge of what the relative antigens are and what, for lack of a better term, we might call antibody is in transplantation immunology, represents a major roadblock in the path of understanding these reactions. Any attempt to investigate systematically anything other than γ globulin that might mediate these reactions should be encouraged, and I think it requires considerable strength of mind.

The α_2 globulin in man, at least, is a frequent acute-phase reaction phenomenon and it is said to be particularly common to nephrosis in general, and especially, I believe, in renal vein thrombosis nephrosis. Perhaps that might account for the particular elevation of this material in West's dogs.

WEST: I thank Fisher and Cooper for their remarks. As the original title of this presentation has implied, we have used fluorescent antibody with results that are as yet uninterpretable and not quite as negative, perhaps, as Fisher's, but they are not as yet a subject for medical publication.

The problem of following red cell agglutination titers, concomitant with the elevation of glycoproteins, would be very interesting. We have not done this. It is deplorable how little is known about the functions or action of this α_2 glycoprotein or of any of the glycoproteins as far as that is concerned; they could very well be related to the red cell agglutination.

RECENT RESULTS OF EXPERIMENTAL TRANSPLANTATION OF PRESERVED LIMBS AND KIDNEYS AND POSSIBLE USE OF THIS TECHNIQUE IN CLINICAL PRACTICE

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Research Institute for Experimental Surgical Apparatus and Instruments, Moscow, U.S.S.R.

The initial attempts to transplant whole organs in experimental dogs were made after the technique of a circular suture of blood vessels had been developed.[1-4]

The first reports of successful kidney autotransplantation in dogs were published in 1902 by Ullman[5] and by Carrel.[4] In the same year Höpfner[6] first replanted a dog's leg; the animal died on the tenth postoperative day from anesthetics administered during the dressing procedure. Carrel,[7-9] Carrel and Guthrie,[10,11] Jianu,[12] Lexer,[13] and other workers who repeated these experiments observed a marked difference in behavior between autografts and homografts. As a rule, dog limb and kidney homografts survive not longer than 2 or 3 weeks following transplantation, while the autografted organs are capable of a prolonged survival. This was demonstrated also by Soviet authors. Khenkin[14] observed 41-day and 122-day survivals of replanted extremities in 2 dogs; several years' survival of dog autografted extremities was observed by Mazajev *et al.*,[15] Vishnevski *et al.*,[16] Petrova,[17] Levchuc,[18] Lapchinsky,[19,20] and others. Long-lived kidney autografts were reported by Voronoy,[21] Shpouga,[22,23] Gritsman and Mazina,[24] Kalinina,[25] Korenevsky *et al.*,[26] and others.

All of these investigators grafted fresh organs immediately after their removal, making every effort to minimize the interval of blood-flow interruption. It is essential, however, as Carrel[27] stated in 1912, to know for how long and under what conditions a removed organ may be preserved for a subsequent transplantation to result in survival and function of the graft.

The limitations to our knowledge in this field and the lack of equipment for preserving and grafting thus far have been major reasons why the method of transplanting autografts capable of a true survival is not used clinically although it has been elaborated in experimental dogs. Nobody has ever succeeded in restoring a completely severed limb to a patient, although a few cases have been reported where successful vascular sutures resulted in restoration of an extremity almost completely separated from the body in an accident.[12,28,29]

To test the possibility of dog-limb replantation after traumatic amputation rather than after sterile surgical procedure, we performed an experiment (March 19, 1954) in which a left extremity of an anesthetized dog was amputated midthigh by a nonsterile axe crushing the bone and soft tissues. Following surgical treatment of the wound with the use of penicillin, the leg was replanted, the total interruption of blood flow being 1 hour and 40 min. The grafted leg, shortened by 35 mm., survived with good restoration of function. The dog has been living for about 6 years since the operation. It runs about, jumps, stands on its hind legs, and rests on the graft, its reduced length being compensated for by a distortion of the pelvis (FIGURE 1).

It is to be supposed that the development of techniques and equipment for preserved-organ transplantation will help to introduce this method into emergency and plastic surgery; it may be used for autografting procedures and later, after solution of the problem of tissue incompatibility, it will facilitate homotransplantation in human cases.

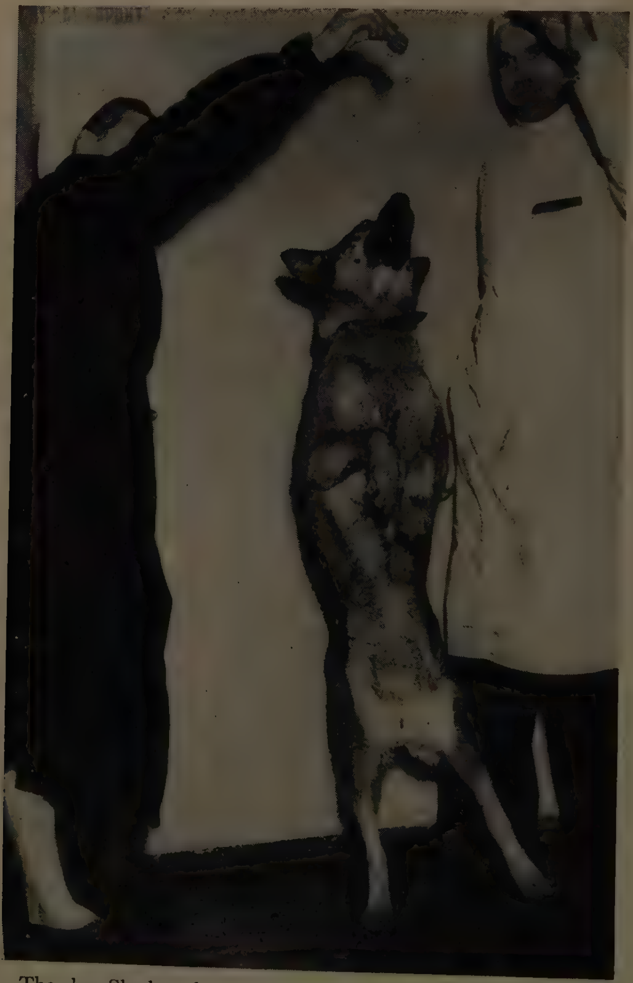

FIGURE 1. The dog Slavka after nonsterile traumatic amputation; this animal, which has been living 6 years since the operation, can stand resting on its replanted left hind leg.

At the same time the preservation can be one of the ways of reducing individual tissue specificity and of circumventing tissue incompatibility. Filatov[30] and other workers observed better survival of preserved corneal grafts than of fresh grafts, explaining this by the presence of biogenic stimulators originating in the preserved cornea. We think this could be explained also by the reduction of individual specificity and, consequently, of tissue incompatibility. Individual variations in the biochemical composition of tissues forming the basis of incompatibility are not likely to originate in the basic structure of organic molecules inherent in each species and firmly established over a prolonged period of evolution. Individual variability refers only to less essential and, possibly, to superficial features of biochemical structures, and these varia-

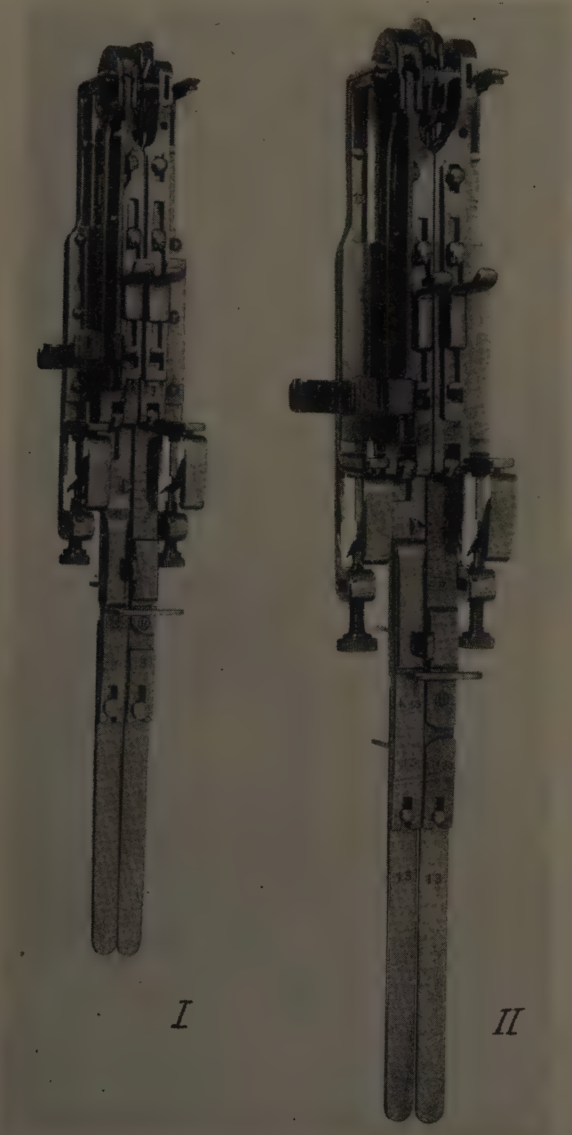

FIGURE 2. A Soviet apparatus for mechanical suture of blood vessels of small and medium size.

tions may be less persistent; therefore, during the mildly necrobiotic process taking place in preserved tissues, they disappear first, that is, individual specificity disappears and a more profound species specificity remains.

The fact of better survival of preserved homografts than of fresh ones, which we pointed out in reports on the transplantation of dental embryos in dogs,[31-34]

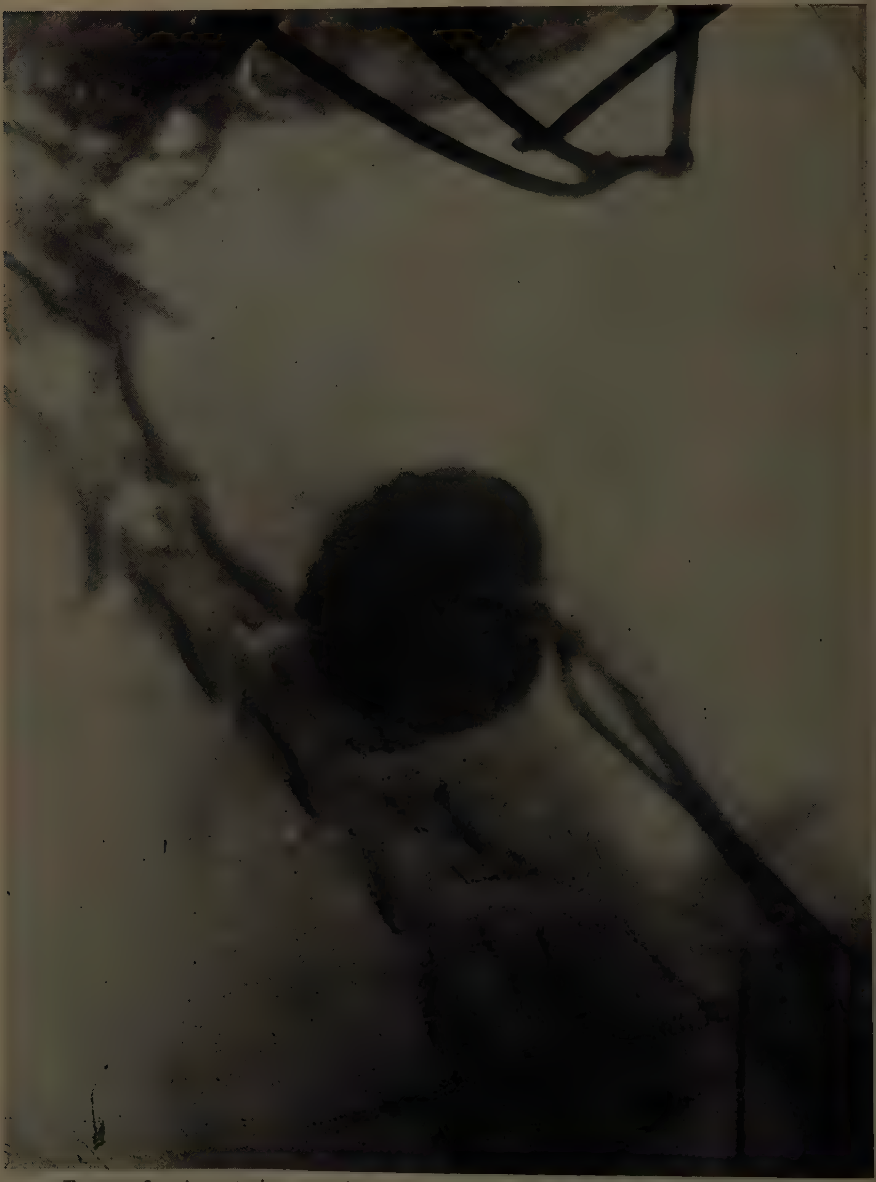

FIGURE 3. An arteriogram of a preserved kidney autografted to a dog's neck.

recently was given a new corroboration in the homografting of rabbit's skin frozen in liquid nitrogen.[35] In this connection it is interesting to study the behavior of grafted preserved whole organs.

Very few published data on preserved-organ transplantation are available. Among them there is not a single description of permanent survival, and the follow-up periods reportedly were of short duration. The first report on experimental preserved-kidney homografting was published by a Roumanian investigator, Avramovici,[36] who preserved kidney in saline solution for 8 to 15 hours. Each of 5 experimental dogs died from diverse causes within 3 to 36 days after receiving the preserved kidney transplant. A Soviet surgeon, Voronoy,[37] was the first to transplant a cadaverous kidney to a human victim of sublimate poisoning. In 1950 he reported[38] on 5 cases of cadaverous-kidney grafting, the kidneys having been preserved for from 12 to 24 days; the grafted kidneys survived not longer than 7 days; some cases showed therapeutic effects in the form of stimulation of the patients' kidneys. Parkinson and Woodworth[39] reported on a successful autograft of a 7-day-preserved goat's kidney, stating that the graft had functioned for 30 days, but not giving any detailed data on its function. Oudot[40] reported on 2 failures of dog kidney autografts following 8-day preservation at 4° C. Lefebvre,[41] who observed the function of homografted kidneys in dogs for as many as 21 days, states that he grafted kidneys preserved for 3 to 50 hours at 0° to 10° C., but gives no details on the graft function. Lastly, Moriwaki[42] grafted dog kidneys preserved for 1 or 2 days in cooled Tyrode's solution; these kidneys functioned for as many as 15 days when autografted and as many as 19 days when homografted. We have found no other data on preserved kidney transplantation.

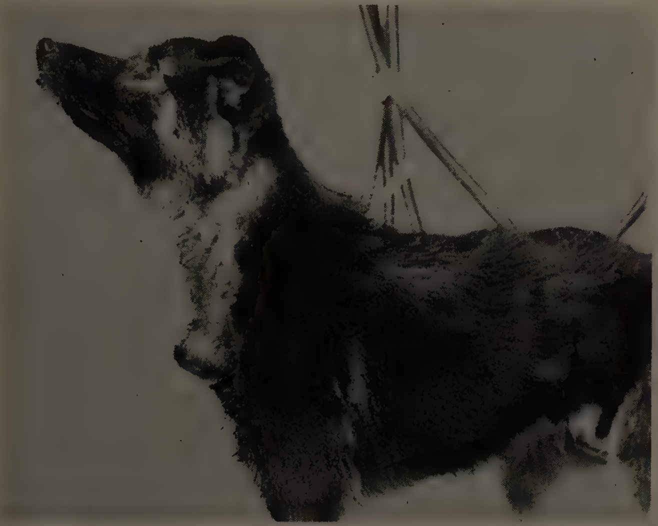

FIGURE 4. The dog Malysh with a kidney grafted to its neck after a 24-hour period of preservation. The intact kidney was removed 2 months after the transplantation, and the animal lived 2 years and 7 months with a single preserved kidney.

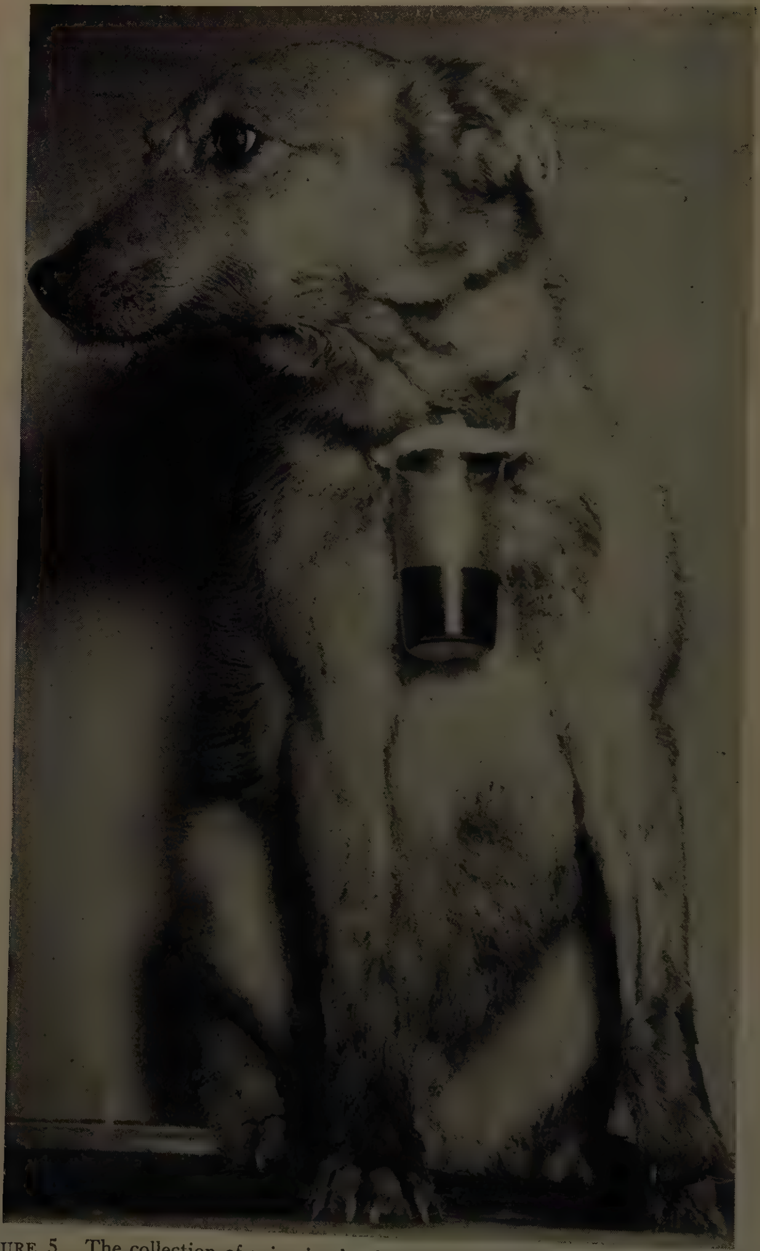

FIGURE 5. The collection of urine in the dog Johnny. The animal lived about 3 years with a single kidney autografted to the neck after 25-hour preservation in the refrigerator unit.

As regards preserved limb transplantation, only one report is available. Blakemore *et al.*,[43] testing their method of vascular anastomosis with a Vitallium prosthesis, replanted dog extremities that had been preserved in crushed ice for as long as 24 hours. They present descriptions of 2 dogs with severe edema of the grafted extremities on the twelfth and twenty-first postoperative days. There are no further references to these dogs in subsequent reports by these authors.

Having at our disposal new surgical instruments developed by the Research Institute for Experimental Surgical Apparatus and Instruments and, in particular, a Soviet-made apparatus for blood vessel circular suture by tantalum staples (FIGURE 2), which is the best means of anastomosing blood vessels, we set up a study with the following aims: (1) to test for the possibility of prolonged preservation of isolated organs prior to their grafting; (2) to develop the most efficient technique of, and equipment for, preserving and grafting isolated organs; and (3) to determine by long follow-up observation the functional capacity of preserved whole organs as compared to that of fresh grafts.

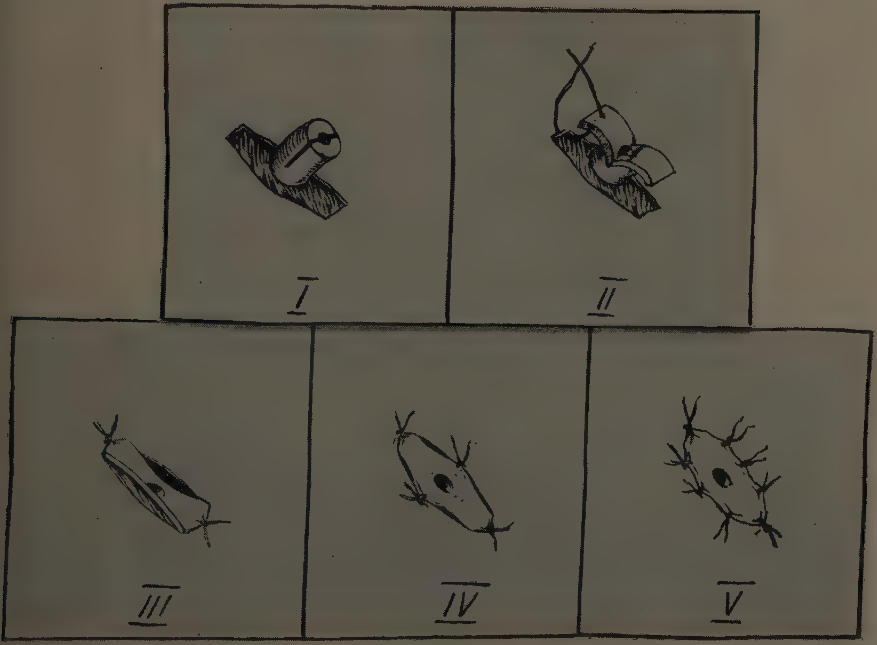

FIGURE 6. The technique of implanting the ureter in the skin; a cicatrix between the skin and mucosa does not constrict the ureteral orifice.

We studied first the viability of tissues of 44 limbs (from 18 dogs) and 67 kidneys (from 34 dogs) preserved by cooling at +2° C. or +3° C. for 3 to 68 hours. From biochemical (Ounik and others) and histological (Lebedeva) findings, and also from tissue culture and vital staining data (Danilova), it was

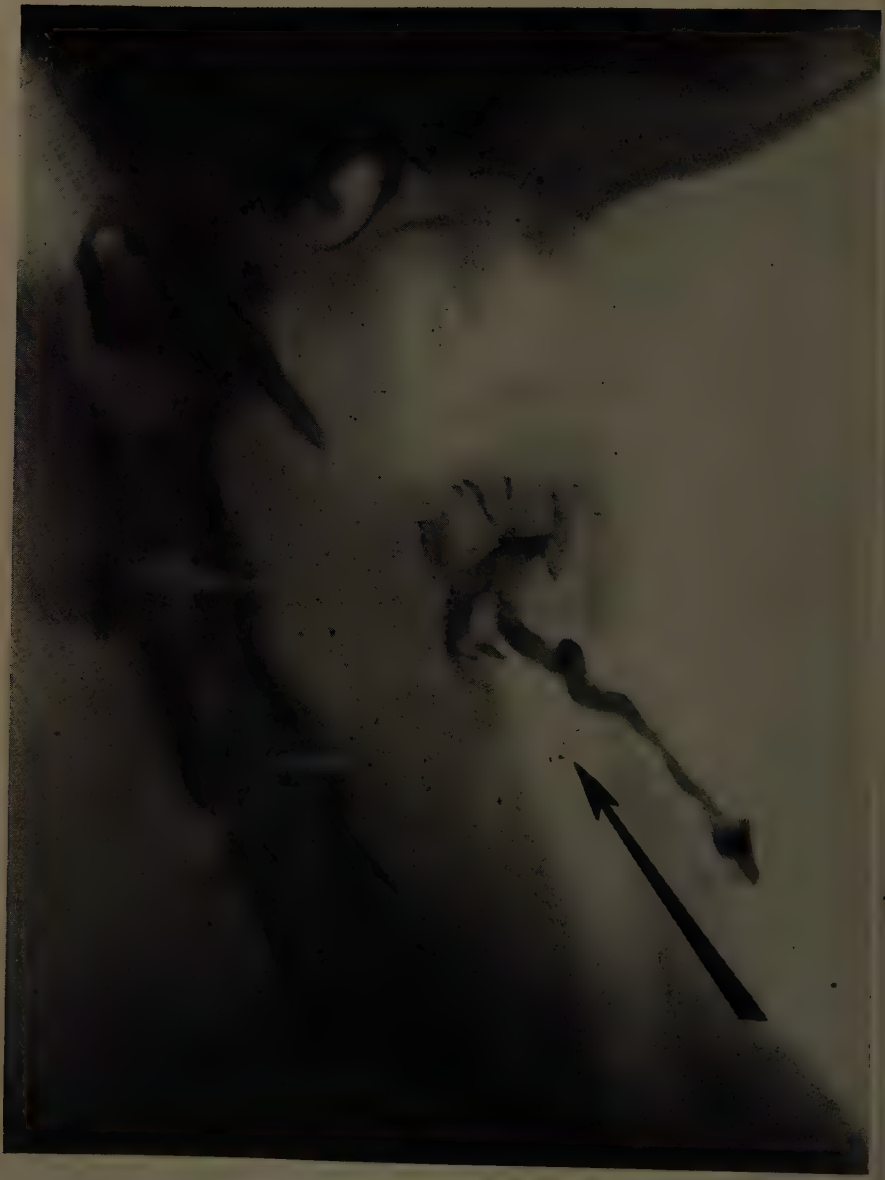

FIGURE 7. A pyelogram of the kidney preserved for 25 hours and autografted to the neck of the dog Meduza; the dog lived after the operation 2 years and 9 months (1 year and 9 months with a single kidney). The pelvis and ureter are not distended. The arrow indicates the shadows of the tantalum staples by which the blood vessels have been sutured.

evident that changes in the tissues of isolated organs, although increasing with the period of preservation, are not significant within the first 24 hours and may be regarded as reversible.[19,20]

In 1953, on the basis of the data obtained, we began to transplant limbs and kidneys preserved at +2° C. and +4° C.* The whole series of experiments (including controls) consisted of 119 organ-grafting procedures with the use of a blood vessel-stapling apparatus. There were 52 kidney transplantations (with permanent functional survival in 16 dogs observed for 3 years and longer) and 67 limb transplantations (25 dogs survived, permanent survival being obtained in 16 dogs observed for 6 years and longer). These experiments showed

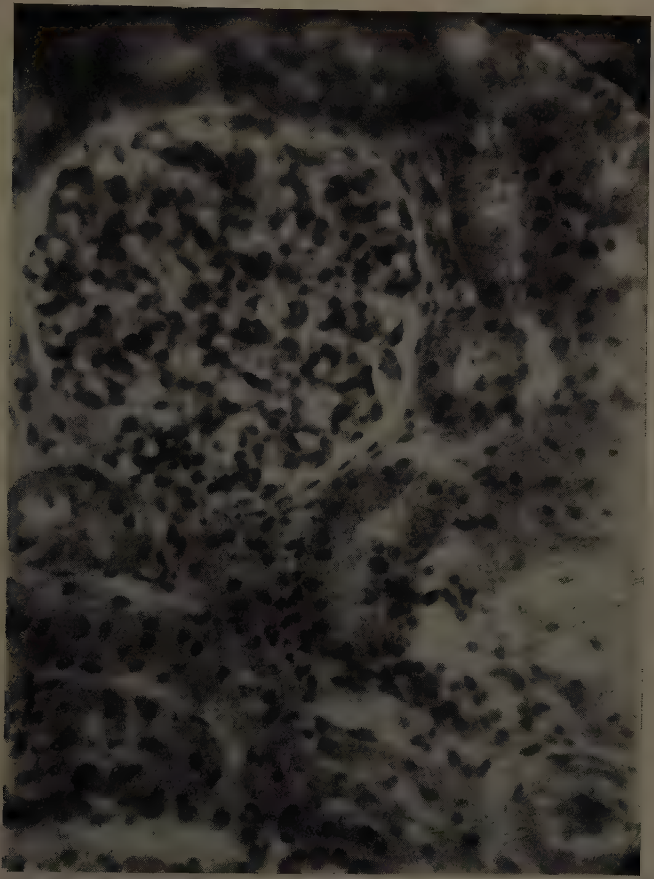

FIGURE 8. Microphoto of the kidney of the dog Ingo 8 months after the transplantation and 6 months after the removal of the intact kidney. The kidney was preserved for 24 hours. Compensatory hypertrophy of glomerular tufts and epithelium of the winding tubules can be seen. × 280.

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* This study was carried out in collaboration with V. F. Victorow, E. B. Gorbovitsky, G. V. Medvedeva, and S. A. Perestoronin.

that a true survival of grafted limbs and kidneys was possible with autografts preserved by cooling at +2° C. and +4° C. for 28 hours (although this interval should not be regarded as a limit).

Transplantation of kidney homografts preserved by the same technique failed to result in a true survival, and the survival time was no better even in 6 experiments on puppies of one litter. We have made no experiments on limb homografts preserved by cooling.

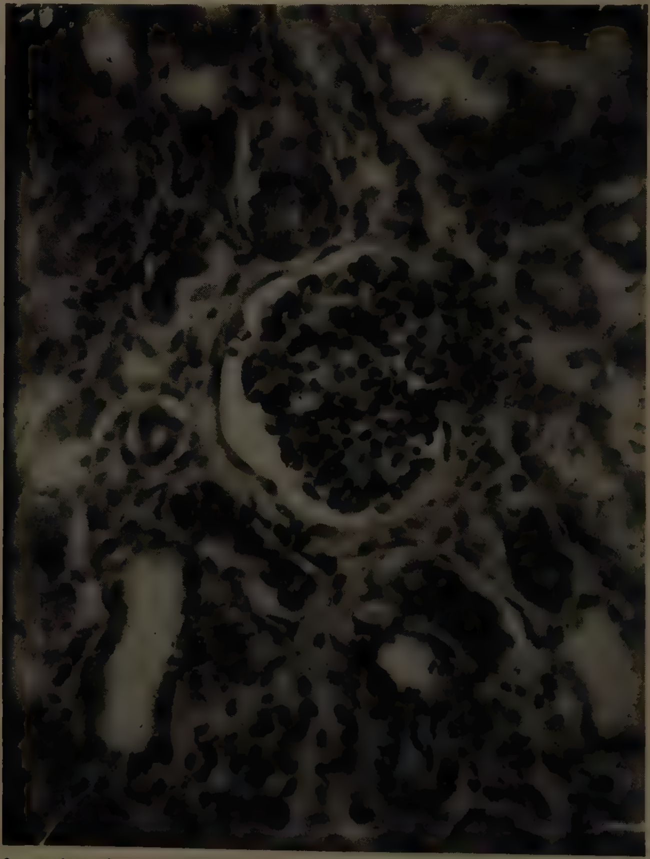

FIGURE 9. Microphoto of a kidney, preserved for 27 hours, 32 days after autotransplantation. Regeneration of winding tubules may be seen. The dog Phantom. ×280.

Preserved kidney autografts transplanted to the dog's neck are capable of a permanent and functional survival both when pre-perfused (with Ringer's solution and heparin or with blood from an individually compatible heparinized donor) and when not perfused. Instead of the usual postoperative anuresis of 1 to 3 days' duration, such autografts are frequently anuretic for 7 to 14 days (one was anuretic for 31 days), even in those experiments in which drops of urine were observed to excrete through the ureter on the operating table, peristalsis of the ureter developing immediately after restoration of blood flow in the transplanted kidney. Only in solitary instances was there no postoperative anuresis; in these the urine excretion began on the first day after grafting. The volume of urine excreted by the grafted kidney gradually increased, but remained less than that excreted by the intact kidney. As shown by the histological studies of Ounik,[44] urine from grafted preserved kidneys at first has very low specific gravity (1.002 to 1.003) and very low creatinine and urea levels; the glomerular filtration rate in these kidneys is much reduced, while no marked changes are observed in tubular reabsorption. This may be explained by the destruction of renal cells during preservation and grafting. Later the renal tissues regenerate, the composition of urine improves, and the urea level increases from 20 to 50 mg./100 ml. to 900 mg./100 ml. Staining with indigo

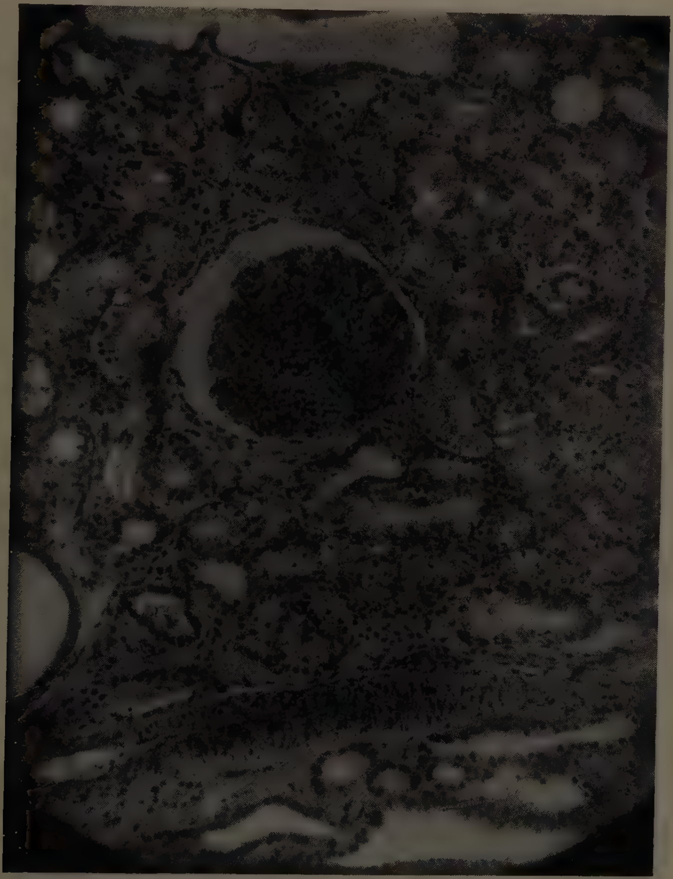

FIGURE 10. Microphoto of a kidney preserved in the refrigerator unit for 25 hours, shown 3 years, 17 days later. The dog lived with this organ as the only kidney over a period of 2 years, 7 months. Hypertrophy of a tuft. The dog Johnny. ×120.

carmine, the ability to respond to the water-milk test with elevated urine excretion, and sometimes even a conditional reflex diuresis of the transplanted kidney (observed in 4 dogs[45]) demonstrate a relatively high functioning of the preserved grafted kidney, although much lower than that of the intact kidney, which usually provides for about 80 per cent of urine excretion.

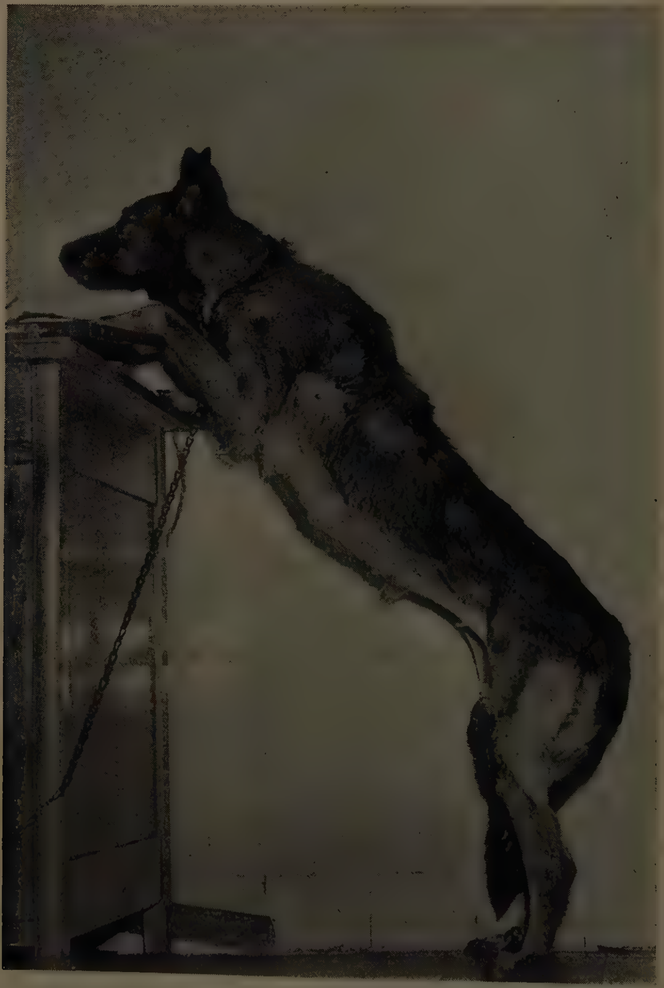

FIGURE 11. The dog Mechta lived more than 5 years after replantation of the left hind extremity preserved for more than 25 hours.

Intra-arterial angiography by Cardiotrast shows a good patency of the arterial network of kidneys transplanted to the neck and an absence of infarcted areas (FIGURE 3).

We carried out a critical biological test to estimate the functional capacity of the preserved engrafted kidneys. In 8 dogs the second intact kidney was removed and the dogs lived with single transplanted kidneys that were able to fulfill the total renal function of the dog for a least 3 years (FIGURES 4 and 5).

These dogs, which are kept in cages in the animal house, behave normally, and the urea in blood plasma remains at upper levels of the normal range, or slightly elevated. They manifest mild recurrent anemia, the number of erythrocytes being 3,000,000, while the hemoglobin is reduced to 40 per cent.[46] This may result from deteriorated conditions of life such as cooling in winter, to which these dogs are very sensitive (as are also the dogs with grafted unpreserved kidneys). When there was deterioration of the general state and an elevated blood urea, the dogs were placed on diets without meat and given penicillin to prevent ascending infection.

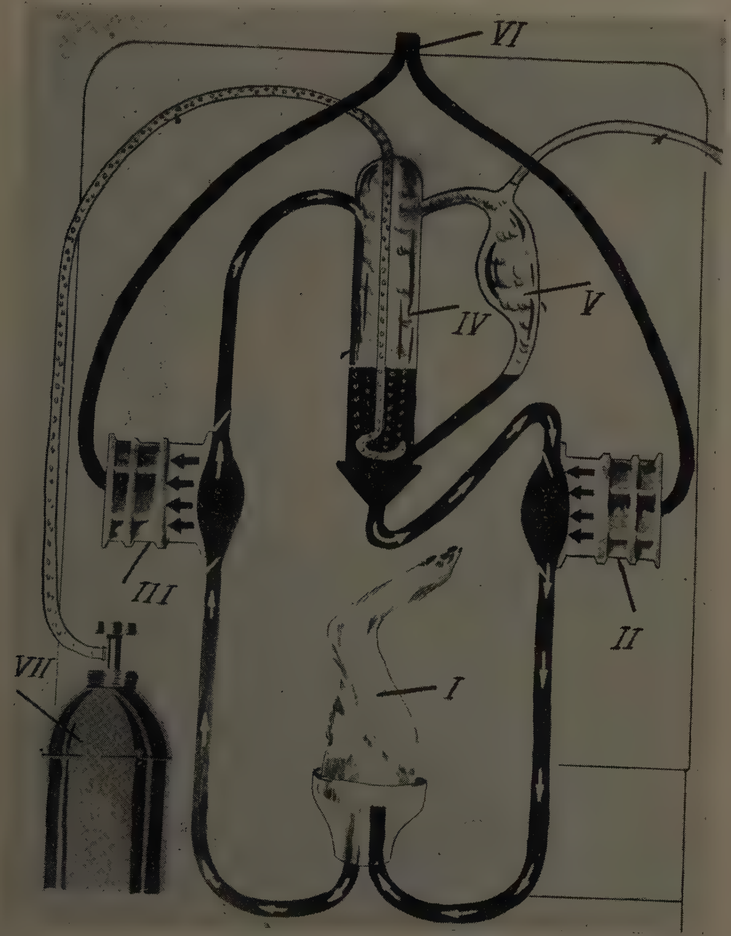

FIGURE 12. Blood-flow circuit in an isolated organ preserved in refrigerator unit prior to its transplantation. I, preserved organ; II, arterial pump; III, venous pump; IV, oxygenator; V, defoamer; VI, hydraulic drive from electric engine; VII, oxygen supply tube.

In some cases animals that lived for a long time died of ascending suppurative pyelonephritis. This was caused by hydronephrosis resulting from inadequate urine removal because of annular cicatrization of the ureteral orifice that had been brought out through the skin. Since we have developed our technique for suturing the ureter into the skin (FIGURE 6)[47], we no longer observe such complications, and pyelograms show normal undistended pelvis and ureter (FIGURE 7).

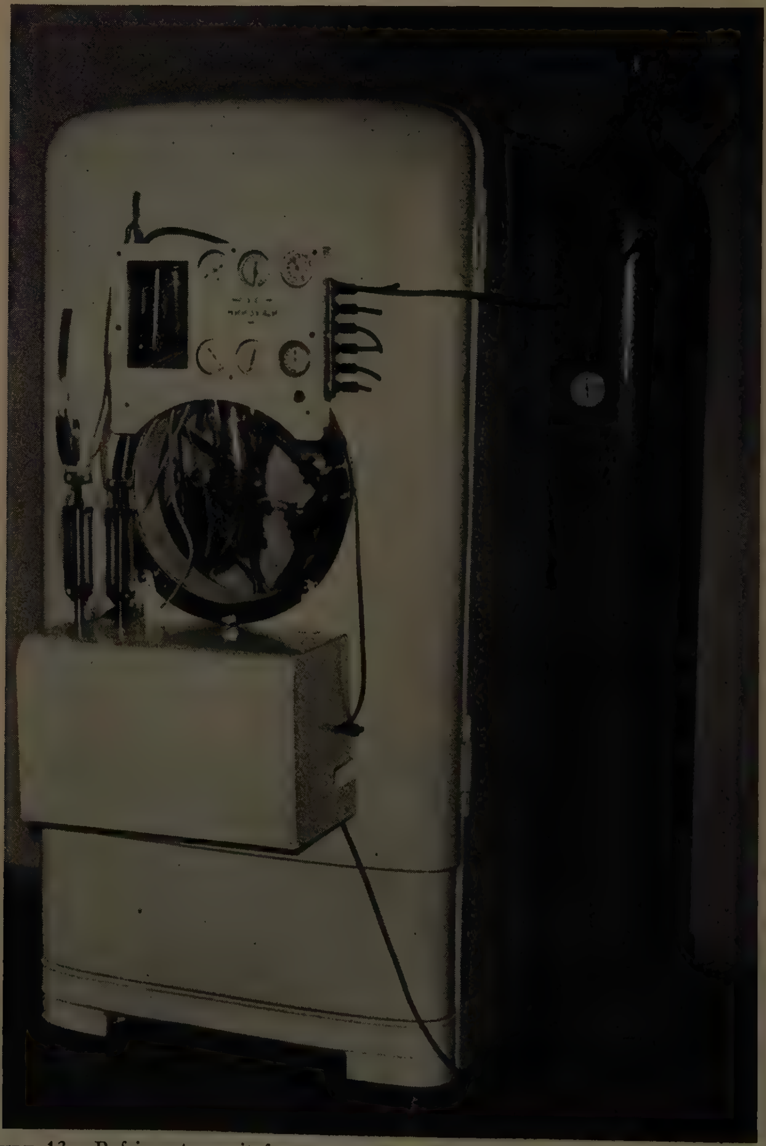

FIGURE 13. Refrigerator unit for preservation of whole organs by artifical circulation.

A remarkable fact was observed during these experiments. In a two-kidney dog the function of the preserved grafted kidney is lower, but within a period of from 1 to 3 days after removal of the intact kidney a spontaneous diuresis and capacity of the graft to remove liquid after a water-milk load become marked. As shown by Ounik,[44] the urea concentration in urine and the glomerular filtration rate likewise increase. The preserved grafted kidney seems to have a higher threshold to hydremia and to nitrogenous wastes accumulat-

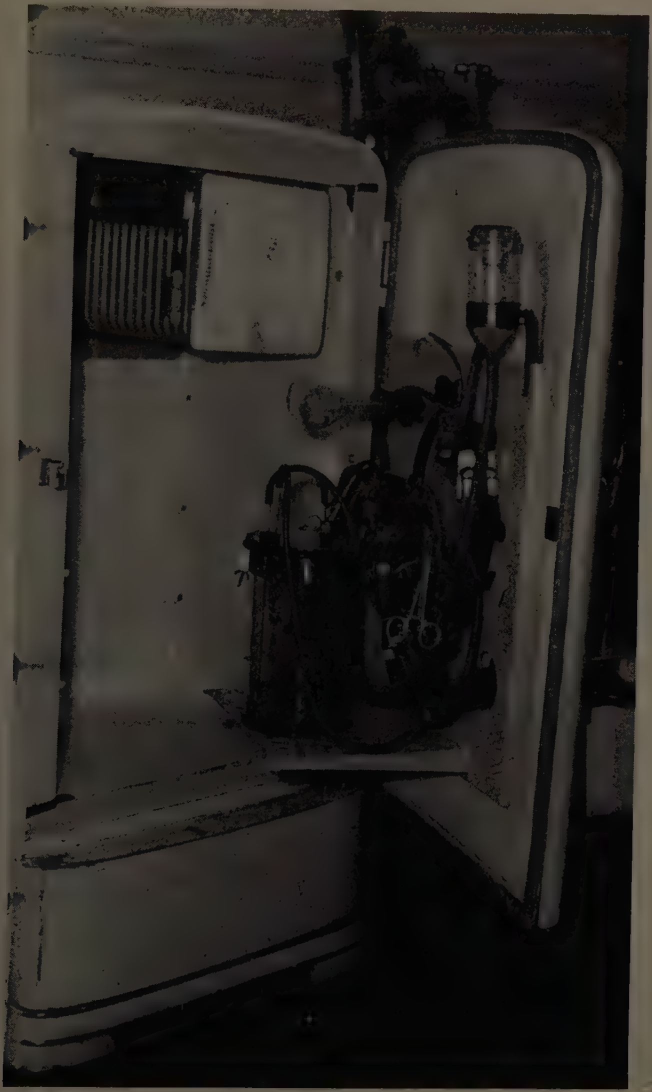

FIGURE 14. A sterile chamber with an isolated kidney preserved in a refrigerator unit with artificial circulation,

ing in the blood, and therefore its function is lower when the intact kidney is present; although it may have a better potential functional capacity, this is not apparent unless the opposite kidney is removed. The remaining single kidney usually has been shown to increase in size and weight. Thus in one dog (Kukla) the preserved grafted kidney weighed 65 gm., while the intact

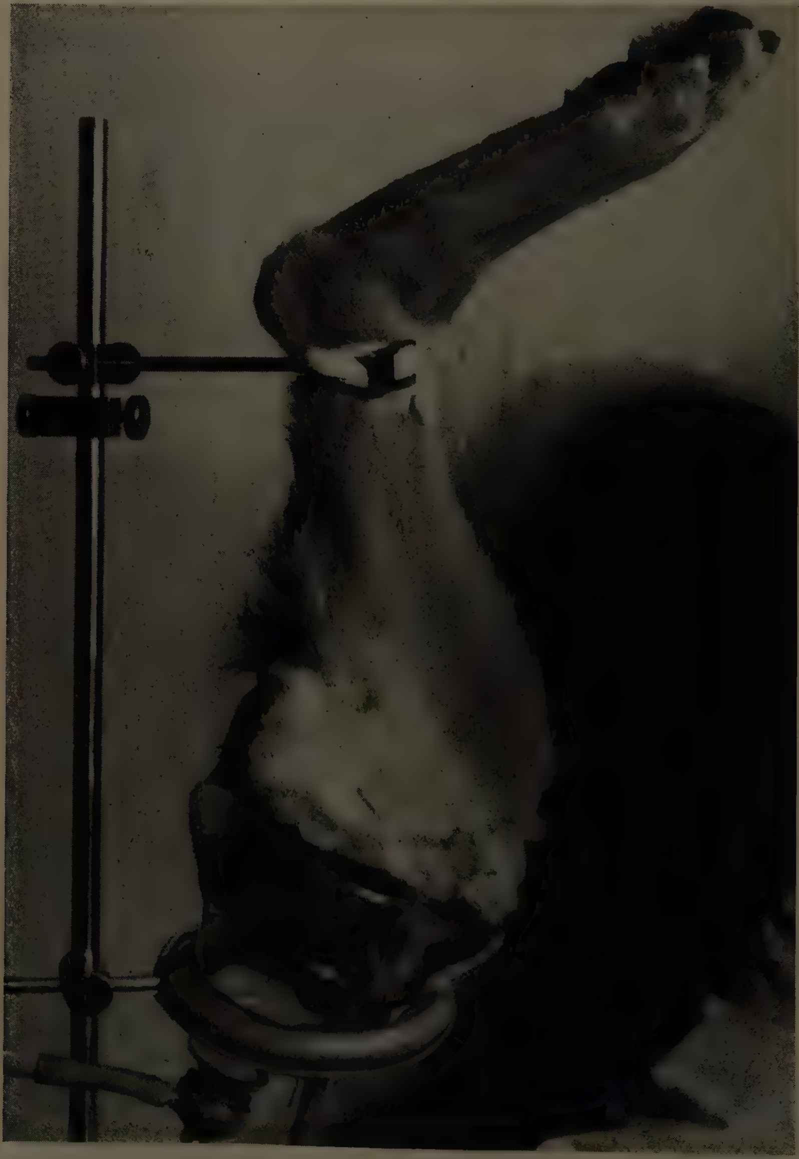

FIGURE 15. An isolated limb in the support is ready for preservation in a refrigerator unit with artificial circulation. The wound surface is covered with a glass cup.

kidney weighed 45 gm.; in another dog (Ingo) the grafted kidney weighed 64 gm. and the intact kidney weighed 30 gm.; in a third dog (Johnny) the graft and intact kidney weighed 67 gm. and 45 gm., respectively. As shown histologically[48] in the dog Ingo, sacrificed 6 months and 12 days after removal of the intact kidney, the grafted kidney, which had been preserved for 24 hours prior to its transplantation, manifested good integrity of renal structures with compensatory hypertrophy (FIGURE 8). A histological specimen of the dog Phantom, taken 1 month after transplantation of a kidney preserved for 27 hours, showed tubular regeneration and general good integrity of the renal tissues (FIGURE 9). Marked compensatory hypertrophy of the glomerular tufts (FIGURE 10) was observed in the dog Johnny 3 years and 17 days after transplantation of a kidney preserved 25 hours (the animal lived 2 years and 7 months with a single kidney).

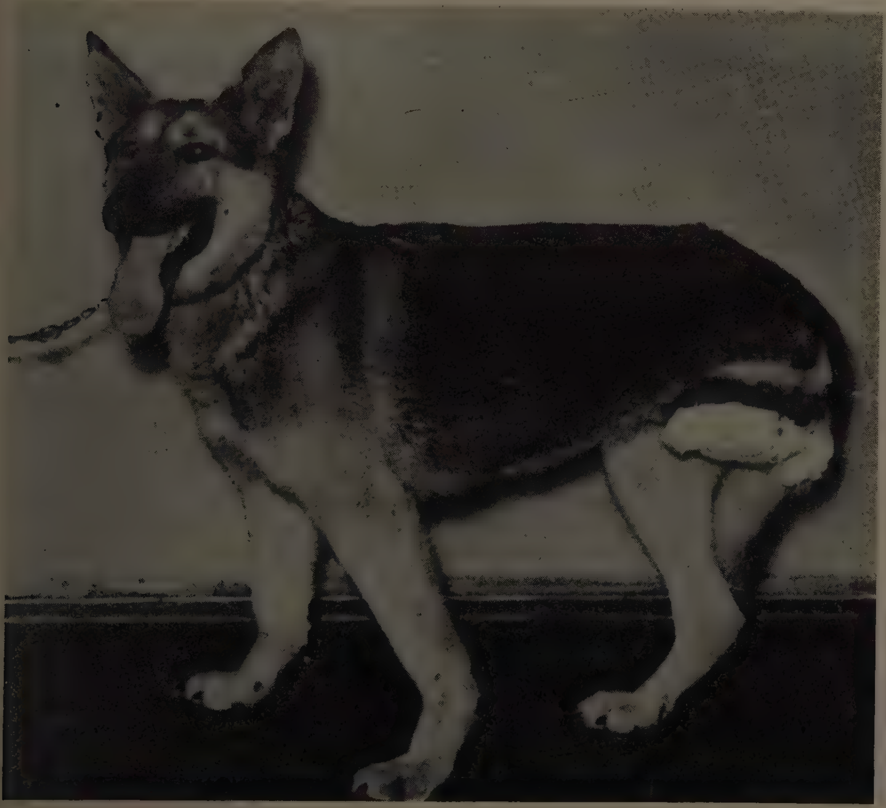

FIGURE 16. The dog Galeta on the second day after the amputation of its left hind leg prior to replantation.

In our experiments with the replantation of extremities preserved for 24 hours and longer by cooling alone we came across a new phenomenon: a toxic effect leading usually to the death of the operated dog. Following the connection of such an extremity to a dog's total blood flow, the animal, which has been quiet and motionless, becomes excited, the respiratory rate and pulse increase, the intestinal peristalsis becomes more intensive, and occasionally yelps and muscle convulsions are observed. The blood pressure gradually falls. Postoperatively, the excitement is replaced by a depression of all vital functions that becomes more and more profound and, within 5 to 12 hours, results in a primary respiratory arrest (sometimes the convulsions occur again before this), and only then the heart stops. When thrombosis develops in the blood vessels of the grafted extremity and the blood flow ceases, the general state of the dog rapidly improves and the animal survives.

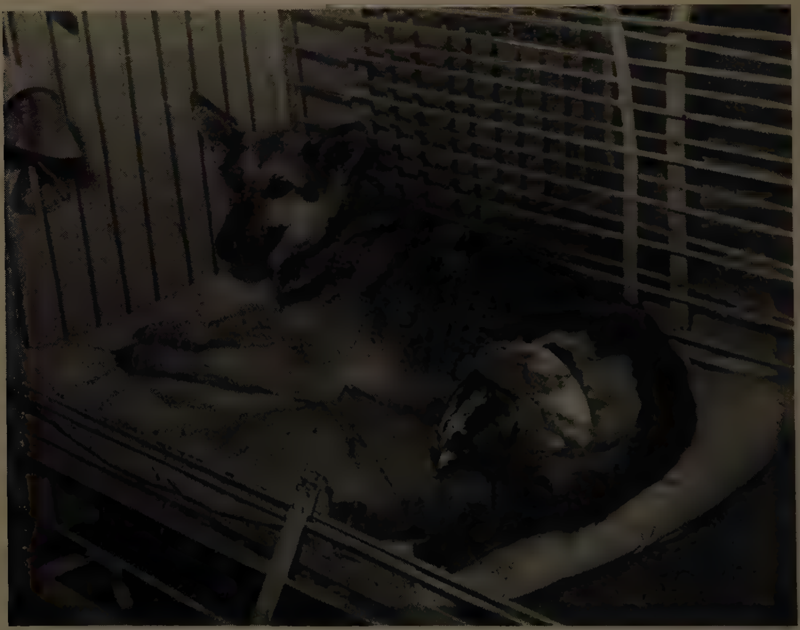

FIGURE 17. The dog Galeta on the sixth day after replantation of the extremity preserved for 25 hours in the refrigerator unit.

In post-mortem examinations and histological studies of lost animals, changes in the parenchymatous organs and cerebral edema can be observed that are proof of toxicosis.[49] It is likely that toxic substances appear in isolated organs during anoxia as a result of impaired metabolism or autolysis. Accumulation of these toxic substances in such large organs as a whole extremity may result in fatal intoxication. This has been confirmed in the experiments performed by Perestoronin,[50] who administered intravenously small doses of perfusate from long-preserved extremities of rabbits and dogs weakened by amputation or toxicosis and observed the death of an animal, when the perfusate was injected into a healthy animal. The biochemical study by Kashchevskaya and Korounzhaya[51] showed an elevated histamine content in the perfusates from extremities long preserved by cooling alone.

The described phenomenon is similar to toxicosis developing after the sudden removal of a long-standing tourniquet, resulting in tourniquet shock, and also is similar to the traumatic toxicosis or crushing syndrome described by Bywaters[52] and others.

The toxic effect never has been observed in the transplantation of long-preserved kidneys, a fact that can be explained by the small size of these organs as compared to a whole body or by the specific features of renal tissue.

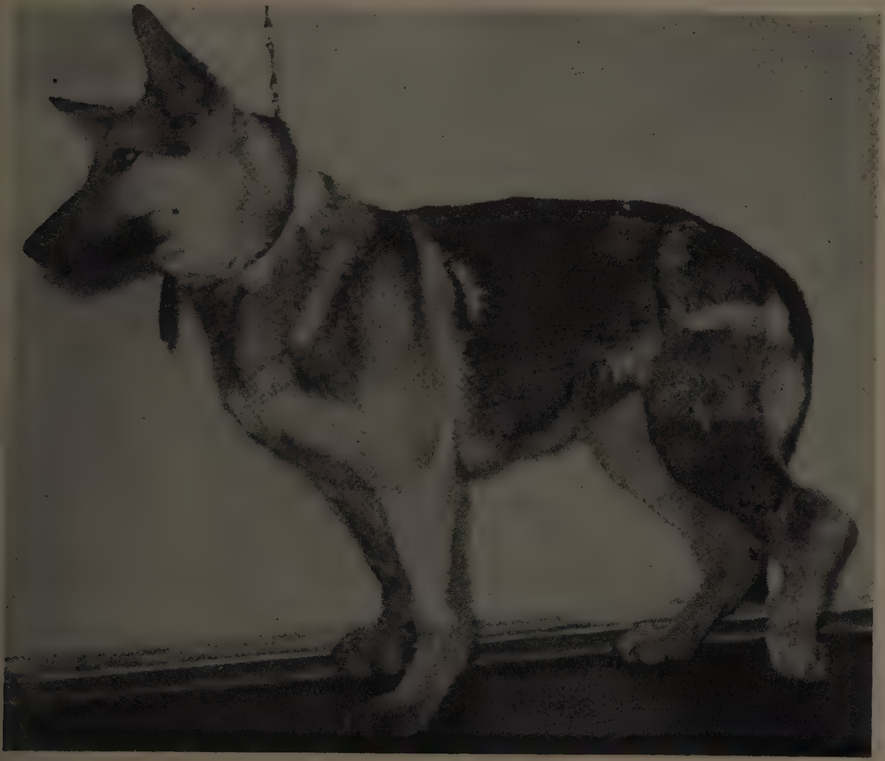

FIGURE 18. The dog Galeta on nineteenth day after replantation of its left hind extremity.

In order to reduce the concentration of toxic matter and to prevent the toxic effect and also to remove minute blood clots from the vessels, the long-preserved limbs, prior to their transplantation, were perfused with Ringer's solution and heparin administered into the femoral artery. By means of this perfusion and various antishock measures we were able to overcome toxicosis and replant an extremity to the dog Mechta after the graft had been preserved by cooling for 25 hours and 11 minutes (FIGURE 11). However, the high mortality rate among dogs with extremities replanted after long preservation by this technique (in particular that of dogs weakened by traumatic amputation) and also the reduced function of the kidneys in the first postoperative period induced us to seek a new and more physiological technique of prior whole-organ preservation.

To prevent anoxia and accumulation of toxic metabolites during prolonged preservation, we suggested the use of artificial circulation in an isolated organ in combination with cooling to reduce metabolism and autolysis (FIGURE 12). In realization of this objective a special refrigerator unit (FIGURE 13) was designed at our institute by G. P. Tjarasov, an engineer; the unit incorporates an S. S. Brukhonenko artificial heart or autojector, a device that has existed in the Union of Soviet Socialist Republics since 1926, when it was first demonstrated at the Second All-Union Congress of Physiologists.[53-55] The entire physiological system and an isolated organ are contained within the unit and may be seen through a window (FIGURE 14). An extremity is placed on the support with a glass cup covering the wound, and a kidney is kept in a special sterile glass chamber (FIGURE 15). The motor and controls are outside the unit.

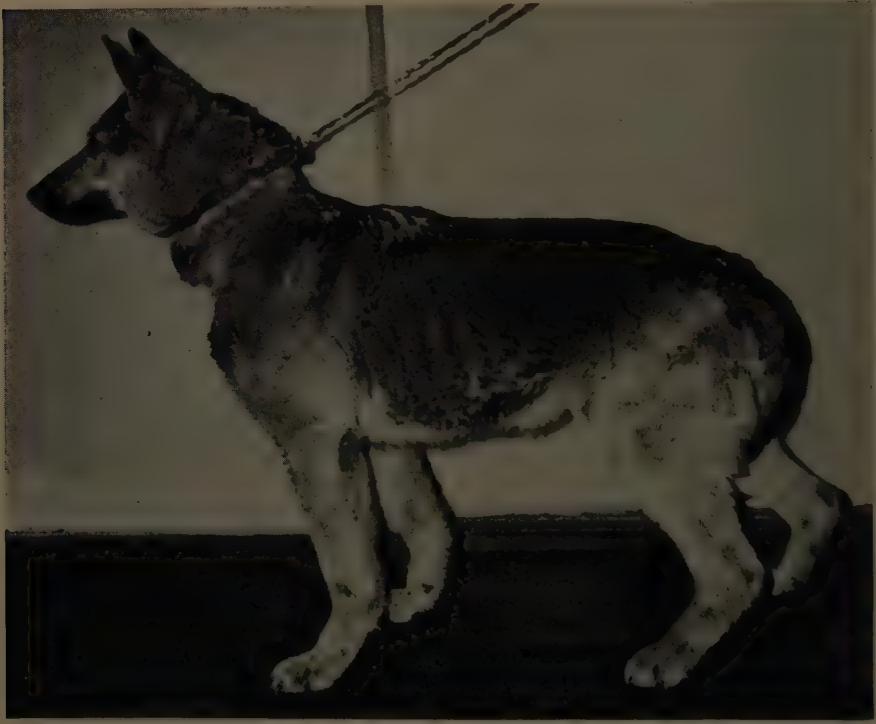

FIGURE 19. The dog Galeta can firmly rest on the grafted leg 6 months after replantation.

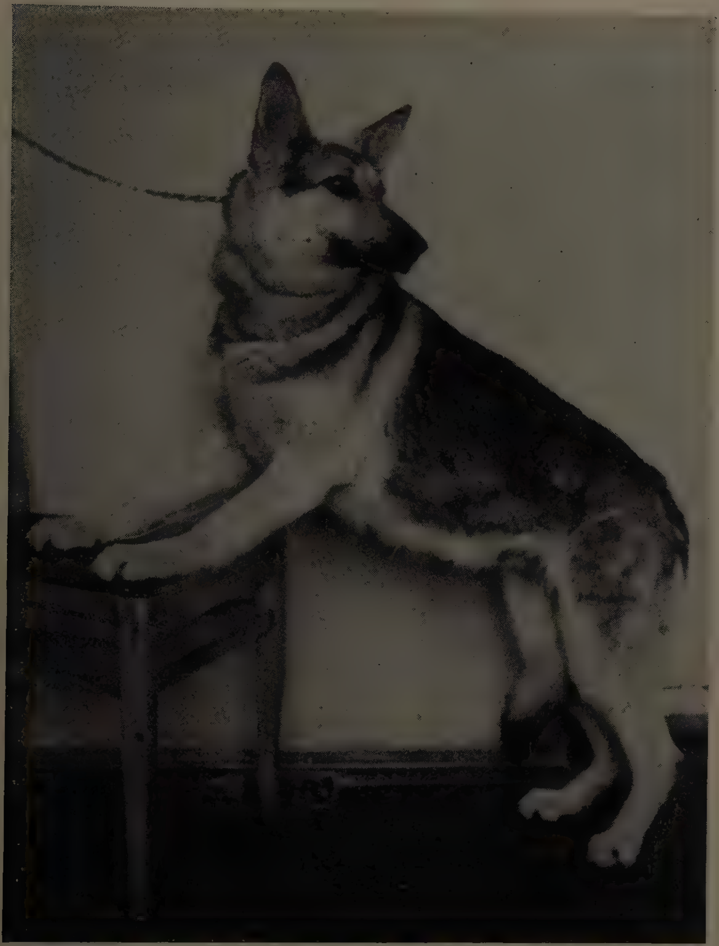

FIGURE 20. The dog Galeta 9 months after replantation of the left hind extremity.

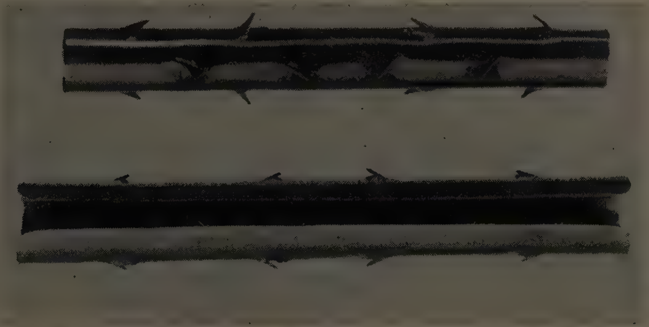

FIGURE 21. Slitted hollow intramedullary pins of stainless steel with sharp teeth for connecting bone ends in replantation procedure.

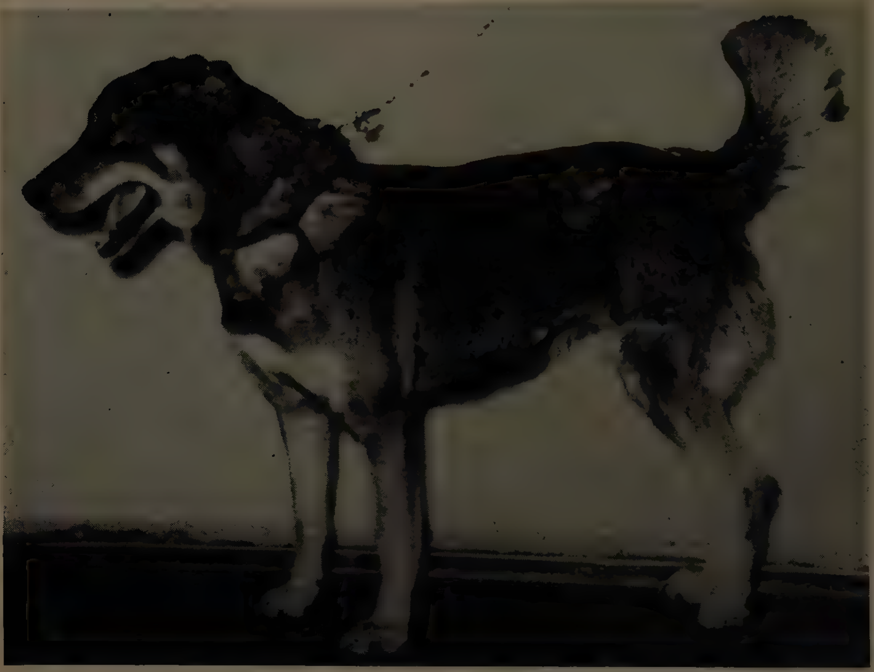

FIGURE 22. The dog Bemka 9 months after replantation of the left hind extremity, preserved for 25 hours and 30 min. in the refrigerator unit. The animal has been living more than 4 years since the operation.

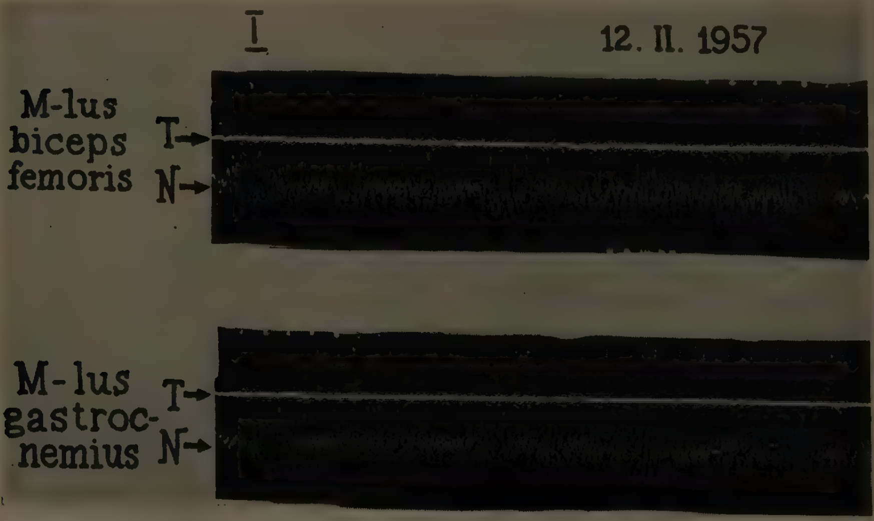

FIGURE 23. An electromyogram of the femoral biceps (*above*) and musculus gastrocnemius of the dog Galeta on day 47 after replantation of the extremity, preserved for 25 hours. T = the graft; N = normal (control). No oscillations can be seen from the grafted femur and shin.

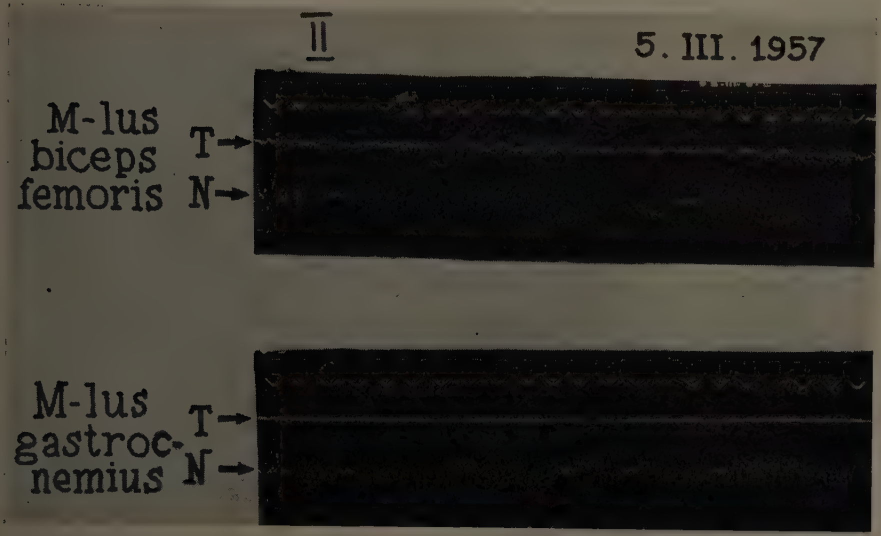

FIGURE 24. An electromyogram of the same dog Galeta on day 69 after replantation of the extremity. Symbols as in FIGURE 23. Slight oscillations appeared in the transplanted femur, but not in the shin.

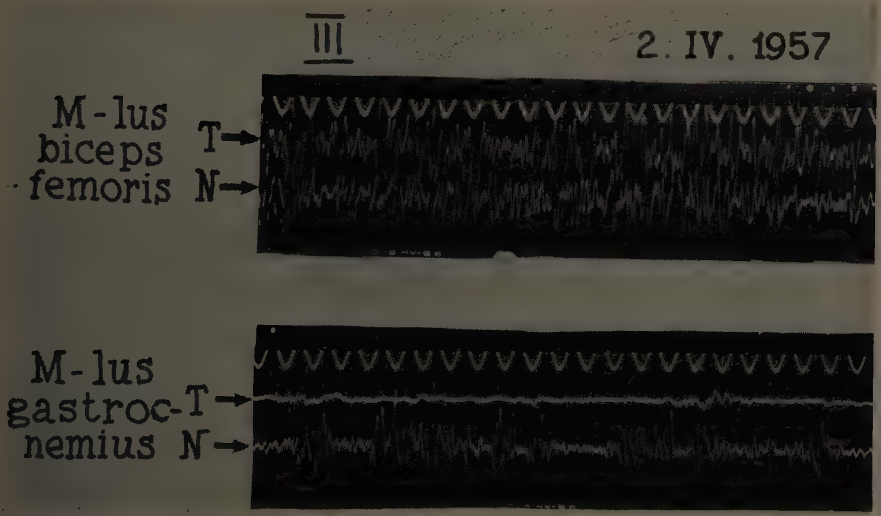

FIGURE 25. An electromyogram of the same dog Galeta on day 98 after replantation of the extremity. Oscillations have been restored in the femur, but they are hardly discernible in the shin.

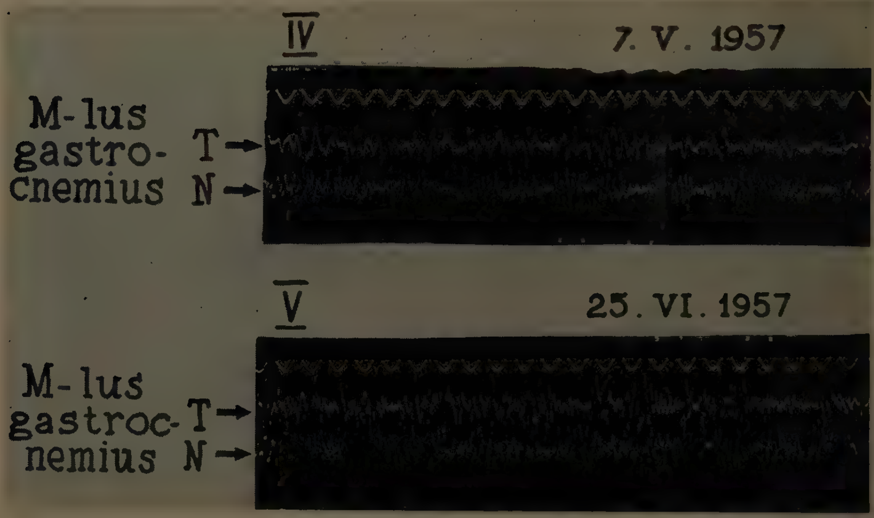

FIGURE 26. Electromyograms of the same dog Galeta on days 133 and 183 after replantation of the preserved extremity. Oscillations have been restored in the femur and shin and are indistinguishable from those in the normal intact limb.

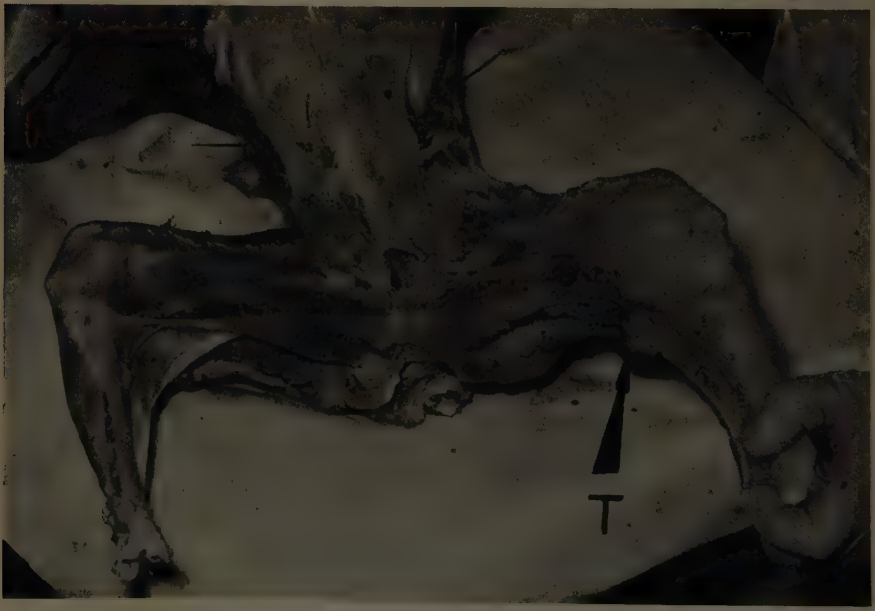

FIGURE 27. Appearance of the grafted (*left*) and intact extremities of the dog Mechta on skinning, more than 5 years after replantation. The graft was preserved for more than 25 hours. No muscular atrophy can be noted.

By means of this unit experiments were carried out in which artificial circulation was used for only 1 hour after the beginning of preservation (by cooled blood with cooling of the organ) and for 1 hour at the end of the preservation period (by heated blood with heating of the organ prior to transplantation). Artificial circulation was not given to a cooled organ.

In 28 experiments on the transplantation of extremities preserved for more than 24 hours in the refrigerator unit by the technique described above, 12 dogs survived without any toxic effects. Of this number 3 died from later complications; in 6 surviving animals the grafted extremities were rejected postoperatively for various reasons, and in only 3 dogs was a prolonged functional survival obtained (FIGURES 16 to 20).

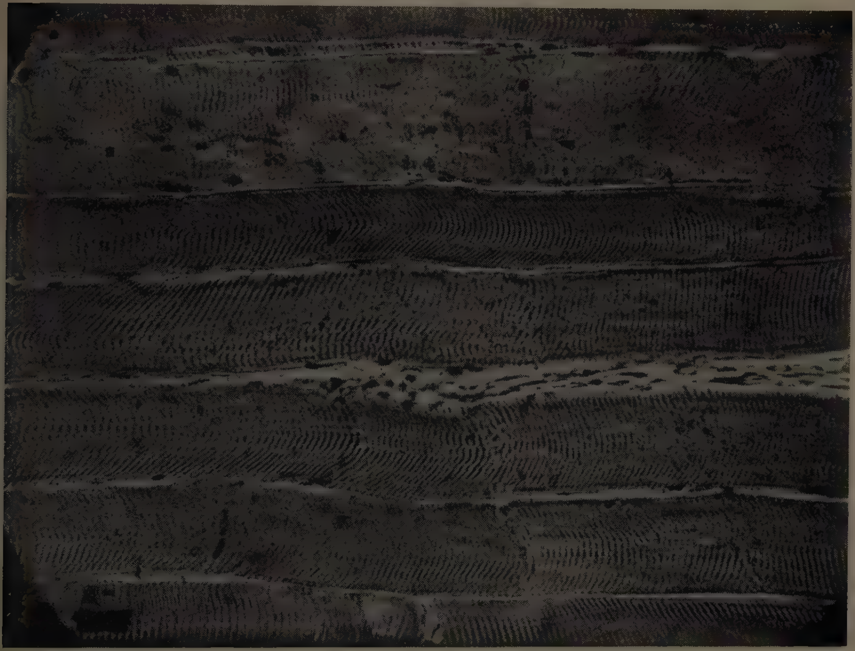

FIGURE 28. A histological specimen from the musculus gastrocnemius of the dog Mechta 5 years and 3 months after replantation of the extremity preserved for more than 25 hours.

In limb-grafting procedures the femur was attached by an intramedullary pin of stainless steel with sharp teeth directed to opposite sides from the mid-line (FIGURE 21); the connection made by this pin was so stable that no plaster bandage was needed postoperatively (FIGURE 22). The continuity of femoral artery and vein was re-established by a mechanical stapler, and the ischiatic and femoral nerves were sutured by hand, with silk 000.

From studies of motor chronaxia, rheobase, motoricity, and other physiological data (E. V. Gourova), it has been found that the restoration of locomotor function in preserved and grafted extremities is comparable to that in extremities grafted without prior preservation. However, the muscular excitability, lost completely in the beginning, reappears only on the eleventh or twelfth day instead of the fourth or fifth, as in the case of fresh extremities. One and one-half months later the muscular excitabilities of both preserved and fresh extremities are about the same. The unconditioned reflex in the shin, which is indicative of functional restoration of the spinal reflex, appears in both instances at about the same time, that is, 3 or 4 months after the operation. The innervation of the digital ends is restored completely in 6 or 8 months after operation.

In 6 dogs the objective evidence of the restoration of the grafted extremity's function was given by electromyography (E. B. Gorbovitsky and J. S. Slavutsky). Thus a left hind extremity was replanted to the dog Galeta December 27, 1956, following preservation in our refrigerator unit for 25 hours and 30 min.; electromyograms of both femoral region (biceps) and shin (musculus gastrocnemius) recorded 47 days after the operation present no oscillations inherent in a normal intact extremity (FIGURE 23). Electromyograms taken on day 69 show slight oscillations in the femoral region, but not in the shin (FIGURE 24). An electromyogram made on day 98 of the femoral region of the grafted extremity presents a normal pattern, but oscillations begin to appear in the shin (FIGURE 25). At 133 days both femur and shin present normal patterns (FIGURE 26). By this time the muscular sensibility and motoricity are restored completely because of the ingrowth of nerve axons through the nerves sutured during replantation. The dogs use the grafted limbs freely, jump, and stand on their hind extremities.

In spite of the denervation of the grafted limb, the growth of hair and claws and the healing of slight skin wounds take place. It is very remarkable that no such chronic trophic ulcers ever have been observed as are seen following the damage of nerve trunks when denervation of an extremity is not complete. The trophic ulcers seem to develop from a chronic area of irritation in the stump of the cut nerve, passing the pathological reflex via cerebral centers to the periphery through nerve anastomoses that are absent in the case of total denervation in replantation conditions.

No marked muscular atrophy developed similar to that following the damage of nerve trunks, despite the restricted movements of the dogs kept postoperatively in cages. The dog Mechta, with an extremity replanted after preservation for 25 hours and 11 minutes, died from sepsis 5 years and 3 months after the operation. On skinning the limbs, the circumference of the femur in both grafted and intact (shortened) extremity were found to be equal (FIGURE 27). Histological sections of the muscles present normal patterns (FIGURE 28). It may be seen from X-ray pictures that the femur had knitted, with formation of a good callus at the intervals comparable to those at which bone union of replanted fresh extremity occurs (FIGURE 29). In X-ray pictures taken with the Cardiotrast patency of the femoral artery sutured mechanically (FIGURE 30) may be seen.

Thus a fact of principal import to both theoretical biology and practical surgery has been established firmly by our studies, namely, a possible maintenance of life in whole compound organs (extremities and kidneys) separated from the body for more than 24 hours. Although no survival has been

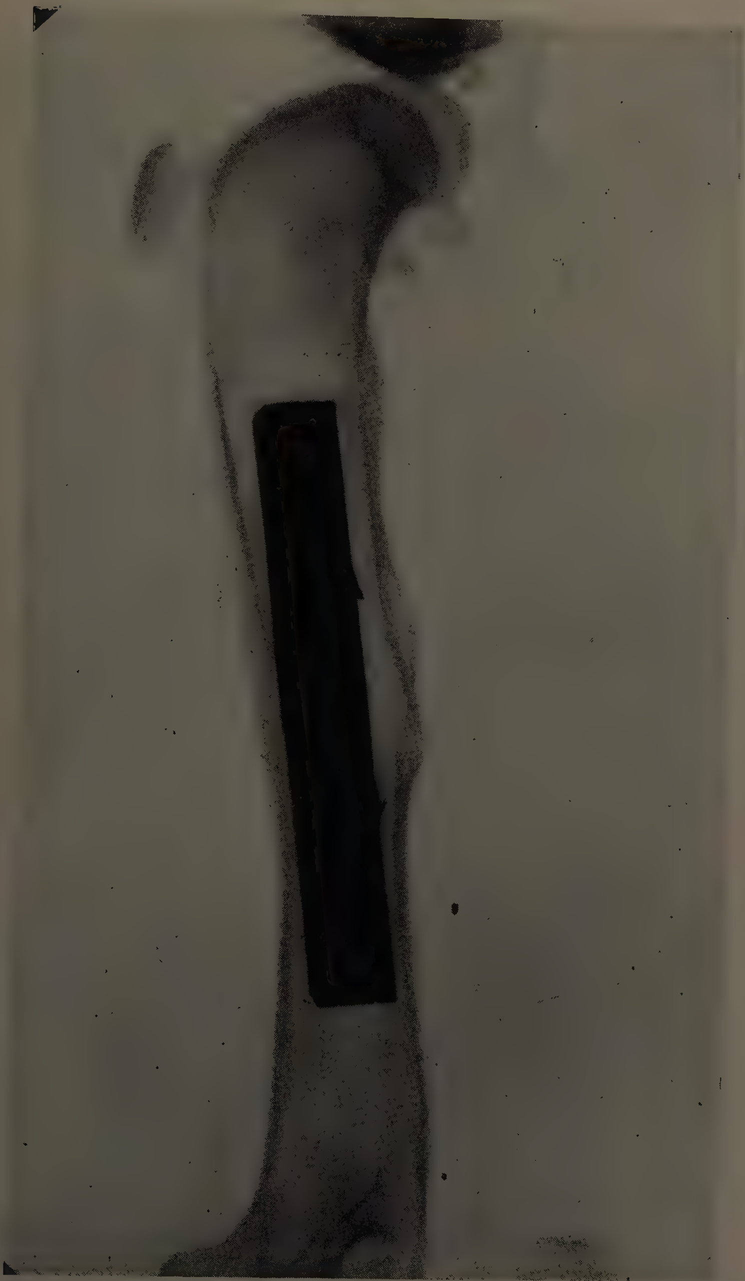

FIGURE 29. An X-ray picture of the dog Slavka after replantation and survival of an extremity.

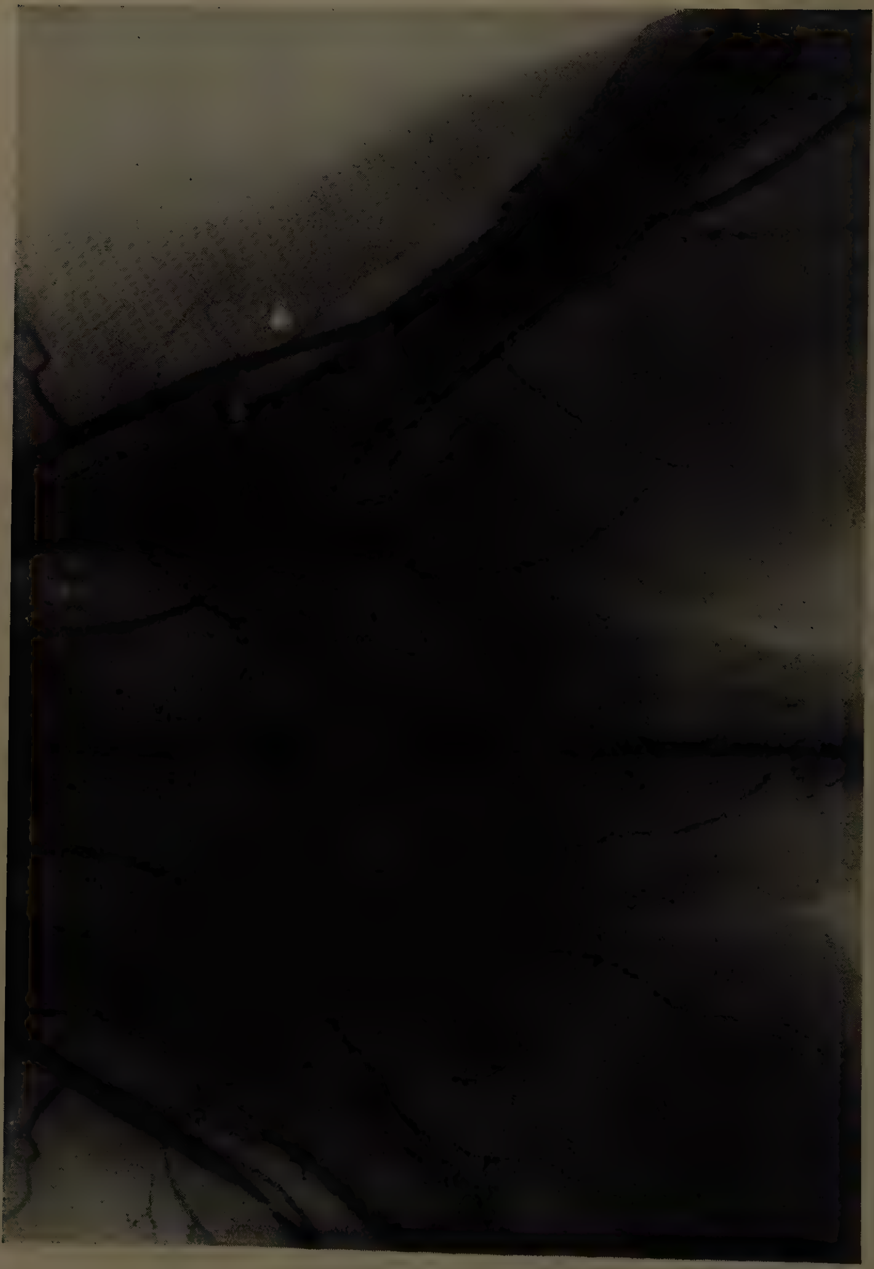

FIGURE 30. An X-ray picture of the vessels of the dog Bemka 4 years and 24 days after replantation of the extremity preserved for 25 hours and 30 min. in the refrigerator unit.

attained following the transplantation of preserved kidney homografts, preserved kidney and extremity autografts are capable of permanent survival and restoration of function.

This opens prospects for the clinical use of the preserved kidney-grafting technique in such cases as the removal of a single kidney in surgical error, when the replantation cannot be performed immediately.

Moreover, these experiments show the possibility of replantation of whole organs following traumatic amputation, especially in such cases of small areas of lesion as trauma from a circular saw and in cases of amputation when the posttraumatic interval is not longer than a few hours. However, the toxic effect observed in the transplantation of long-preserved limbs indicates the dangers of the method and the necessity of developing further the preservation technique.

Following the development of a reliable technique of circumventing immunological incompatibility of tissues during grafting procedures, such a method may be used in homotransplantation as well.

References

1. Murphy, G. B. 1897. Resection of arteries and veins injured in continuity end to end suture. N. Y. Med. Record. **16:** 1073.
2. Payr, E. 1900. Beiträge zur Technik der Blutgefäss- und Nervennahnebst Mittheilungen über die Verwendung eines resorbirbaren Metalles in der Chirurgie. Arch. klin. Chir. Langenbecks. **62**(1): 67–93.
3. Payr, E. 1901. Weitere Mittheilungen über Berwendung des Magnesiums bei der Naht der Blütgefässe. Arch. klin. Chir. Langenbecks. **64** (3): 722–740.
4. Carrel, A. 1902. La technique operatoire des anastomoses vasculaires et la transplantation des viscères. Lyon méd.
5. Ullman, E. 1902. Experimentalle Nierentransplantation. Wien. klin. Wochschr. **15**(11): 281–282.
6. Höpfner, E. 1903. Über Gefässnaht, Gefässtransplantationen und Replantation von amputierten Extremitäten. Arch. klin. Chir. Langenbecks. **70:** 417–471.
7. Carrel, A. 1905. The transplantation of organs. J. Am. Med. Assoc. **45**(22): 1645–1646.
8. Carrel, A. 1908. Further studies on transplantation of vessels and organs. Proc. Am. Phil. Soc. **47:** 677–696.
9. Carrel, A. 1909. Doppelte Nephreetomie und Reimplantation einer Niere. Arch. klin. Chir. Langenbecks. **88**(3): 378–411.
10. Carrel, A. & C. Guthrie. 1905. Functions of a transplanted kidney. Science. **22:** 473.
11. Carrel, A. & C. Guthrie. 1906. Anastomosis of blood-vessels by the patching method and transplantation of the kidney. J. Am. Med. Assoc. **47:** 1648.
12. Jianu, J. 1913. Beiträge zum Studium der Transplantationen. Arch. klin. Chir. Langenbecks. **102**(1): 57–65.
13. Lexer, E. 1919. Die Freien Transplantationen. 1. Stuttgart, Germany.
14. Хенкин В. Л. 1940. Обратное пришивание отрезанной ноги. Тр. Ростов. Тос. Мед. Инст. УП; : 1–5.
15. Мазаев П. Н. Чепов П. Н. и Коваль М. А. Кровообращение пересаженной конечности. Хир. **5:** 48–55.
16. Вишневский А. А., Мазаев П. Н., Чепов П. М. и цр. 1952. Проблемы пересацки органор. Хир **8**; 5–12.
17. Петрова Н. П. 1954. Механический сосуцистый шов в экснерименте. Автореф. канд. дисс. **1:** 1–14.
18. Левчук Г. А. 1954. Некоторые данные о пересадке/реимплантации/конечности у собак. Вр. Дело **10**; 909–912.
19. Лапчинский А. Г. 1955. Пересадка консервированных целых органов в эксперименте и значение новой аппаратуры для этой цели. Хир. **4:** 54–59.
20. Lapchinskij, A. G. 1956. Il trapianto sperimentale di organi interi conservati e l'umportanza di una nuova attrezzatura per la sua execuzione. Rass. med. Sov. **1:** 35–41.

21. Вороной Ю. Ю. 1930. Демонстрация собаки с пересаженной с помощью сосудистого шва почкой под кожу шеи. Труд. 1У Всеукр. С″езда Хир. Нов. Хир. Архив. **23**; 200–203.
22. Шпуга Г. М. 1937. К демонстрации функции пересаженных на шею почек. Сб. докл. У1 Всес. С″езда физиол. биохим. и фарм. : 726–729.
23. Шпуга Г. М. 1956. Сосудистый шов и пересадки почек. Экспер. Хир. **1**: 19–27.
24. Грицман Ю. Я. и Мазин Ф. В. 1952. Исследование мочевыделительной функции аутопластически пересаженной почки. Бюлл. Эксп Биол и Мед. **12**.
25. Калинина Т. В. 1955. К вопросу о пересадке почки. Урол. **1**; 43–49.
26. Кореневский Л. И., Левчук Г. А. и Шведкова—Роме Т. С. 1956. Об аутотрансплантации почки. Вр. Дело **3**; 277–280.
27. Carrel, A. 1912. The preservation of tissue and its application in surgery. J. Am. Med. Assoc. **59**(7): 523–527.
28. Андросов П. И. 1952. Механический шов при ранении кровеносных сосудов. Воен. Мед. Журн **2**; 51–56.
29. Базилевская Ю. В. 1958. Механическом сосудистом скрепочном шве. Вопросы Травмат. и Ортоп. **5**: 124–133.
30. Филатов В. П. 1937. От оптической пересадки роговицы к лечебной трансплантации тканей. Арх. биол. наук. **50**: 21–35.
31. Lapchinsky, A. G. & A. A. Malinowsky. 1940. An attempt at experimental homoplastic transplantation of teeth in the dog. Compt. rend. sci. URSS. **28**(8): 750–753.
32. Lapchinsky, A. G. & A. A. Malinowsky. 1943. Further experiments on substituting lost teeth by means of homoplastic transplantation of tooth anlages. Compt. rend. sci. URSS. : 178–180.
33. Лапчинский А. Г. 1940. Гомопластические пересадки конечности у крыс, как доказательство возможности приживления гомопластических трансплантатов у млекопитающих. Хир. **10**: 15–26.
34. Lapchinsky, A. G. 1941. On homoplastic transplantation of organs in mammals. Trav. inst. cytol. hystol. et embryol. **1**(1): 183–207.
35. Лапчинский А. Г. и Лебедева Н. С. 1959. Опыт пересадки кожи кроликов замороженной до—196град. С.
36. Avramovici, A. 1924. Les transplantation du rein. Lyon chir. **21**: 734–757.
37. Вороной Ю. Ю. 1934. К вопросу о блокаде ретикуло-эндотелиального аппарата у человека при некоторых формах отравления сулемой и о свободной пересадке почки. Тр. Всеукр. Инст. Неотложн. Хир. и перелив. крови. **1**: 221–234.
38. Вороной Ю. Ю. 1950. Пересадка консервированной трупной почки, как метод биостимуляции при тяжелых. нефритах. Вр. Дело **9**: 814–816.
39. Parkinson, D. & H. Woodworth. 1947. Observations on vessel and organ transplants. Exptl. Med. Surg. **5**: 49–61.
40. Oudot, T. 1948. Transplantation renale. Press med. **56**: 319–320.
41. Lefebvre, L. 1952. Traitement experimental de l'uremie par transplantation rénale. J. urol. med. et chir. **58**(5): 225–231.
42. Moriwaki, F. 1956. J. Wakayama Med. Soc. **6**(3): 533–555.
43. Blakemore, A., J. Lord, & P. Stefko. 1943. Restoration of blood flow in damaged arteries; further studies on a nonsutural method of blood vessel anastomosis. Ann. Surg. **117**(4): 481–497.
44. Уник В. И. 1955. Функция консервированных аутопластически перссаженных почек. Бюлл. Экспер. биол. и мед. **8**: 22–26.
45. Даниельсон А. К. 1957. Особенности Условно-рефлекторной деятельности консервированной аутопластически пересаженной почки. Экспер. Хир. **4**: 44–48.
46. Шишкина И. Д. 1959. Изменения морфологического состава периферичес-кой крови при аутопластических пересадках консервированных органов у собак. Сб. Проблемы пересадки и консервации органов и тканей. : 269–271.
47. Лапчинкий А. Г. 1956. О способе введения в кожу трубчатых протоков. Труд. Конфер. 80–летия В. П. Филатова : 295–296.
48. Лебедева Н. С. 1959. Морфология консервированных почек при аутопластике. Сб. Проблемы пересадки и консервации органов и тканей : 263–266.
49. Савченко Е. Д. 1959. Патолого-анатомическая картина токсикоза при пересадке консервированной конечности у собак. Сб Проблемы пересадки и консервации органов, и тканей : 262–263.
50. Пересторонин С. А. 1958. О накоплении ядовитых веществ в тканях изо-

лированных органов, консервированных в условиях аноксии. Физиология и патология дыхания, гипоксия и оксигенотерапия. Киев. : 463–465.

51. Кащевская Л. А. & Хорунжая К. Ю. Динамика некоторых биохимических показателей крови при пересадке консервированных целых органов у собак. Тез. 1 Всес. Конфер. по проблеме тканев. несовм. и. трансп. органов и тканей. Москва : 59–60.
52. Bywaters, E. 1944. Ischemic muscle necrosis, crushing injury, traumatic edema, the crush syndrome, traumatic anuria, compression syndrome. J. Am. Med. Assoc. **124**(16): 1103–1109.
53. Брюхоненко С. С. 1926. Опыты по изолированию головы собаки. Труд. П Всесоюзн. с″ езда физиологов : 289–290.
54. Brukhonenko, S. S. 1929. Circulation artificielle du sang dans l'organisme entier d'un chiens avec coeur excl. J. Physiol. Pathol. gén. **27**: 1.
55. Брюхоненко С. С. Щербакова Т. Т. Перестoронин С. А. Лапчинский А. Г. и др. 1959. Опыт применения аппарата искусственного кровообращения/СБ-з/Для полного выключения сердца. Сб. Нов. хир. аппараты и инструм. и опыт их примен. Ш Москва : 43–50.

Discussion of the Paper

Blair O. Rogers: The question has arisen as to nervous regeneration in the retransplanted limb. I emphasize the fact that I inspected the legs of these animals fairly closely. There does not seem to be any obvious atrophy or wasting of the muscles in these limbs although each dog seems to have a slight instability of its leg when it runs too fast up and down the hall of the institute, especially when it tries to stop short. The leg then occasionally gives way under the momentum of the dog, but there is also a marked stability of the same leg in other positions, for example, when the dog walks at a normal pace or stands only upon his two hind legs, one of which is the retransplanted limb. If we can accept the electromyographic reports shown by Lapchinsky, and there seems to be no reason why we should doubt them, they certainly seem to follow the classical teachings of neurosurgeons, for example, that a period of 4 to 6 months or longer must elapse before signs of adequate motor innervation return to the muscle groups supplied by a severed peripheral nerve. Lapchinsky's serial electromyographs demonstrate that motor activity of the major most distal muscle, namely the gastrocnemius, farthest from the amputation site, did not return to fairly normal electromyographic patterns until 133 days or approximately 4½ months had elapsed.

Question: Did you say whether Lapchinsky does or does not suture the nerve?

Rogers: He sutures both the ischiatic and femoral nerves by hand with 000 silk.

David M. Hume: I think Rogers has touched upon one of the most fascinating points of this presentation, namely, that, under these circumstances, one does obtain an apparent nerve regeneration. This, I believe, might not have been predicted prior to knowing of this study and of the photographs accompanying it.

There are a number of ways that one can theoretically or actually utilize to preserve kidneys for varying periods of time. The paper we have just heard has dealt mostly with short-term preservation, up to 24 or 48 hours. One can place the kidney in a pump arrangement of some kind, as also demonstrated in this paper, and perfuse the organ with blood or other solutions for short periods to maintain it.

One can transfer the kidney from animal to animal for perfusion purposes, as has been done by various workers in the United States, or one can place the kidney in a container and transfer various animals to the container, each perfusing the kidney for 24 hours at a time. One can maintain the kidney for a prolonged period of time by the latter technique.

Theoretically, one can utilize other methods of oxygenation or other methods of energy transfer to preserve a kidney. One need not necessarily use blood. There are perhaps other substances that one could utilize to maintain its metabolism for short periods of time. One can alter the metabolism with low temperatures, as has been demonstrated here.

Most of these methods are essentially techniques suitable for fairly short-time preservation. Egdahl and Harris in our laboratory wished to investigate the possibilities of glycerine perfusion in the maintenance of the kidney at very low temperatures for long periods of time. As the first step in this study, they perfused the isolated kidney in the dog with various concentrations of glycerine (10, 15, and 20 per cent) and then removed the remaining kidney following perfusion of the kidney to be grafted to see whether kidney function had been impaired by the glycerine. In all cases kidney function failed completely, so that this does not appear to be a good technique for prolonged preservation.

Apart from the scientific interest which this kind of work generates, the main purpose of this type of preservation lies in the natural desire to use cadaver source material for transplanted tissues and organs.

Certainly, our own experience with the use of cadaver kidneys in the human is such that by the time we get the kidney from the cadaver, the donor had already been in an agonal phase of dying for some time, and the kidney has thus been anoxic for some period. Then, by the time you procure the kidney and remove it, even if you transfer it to the patient as quickly as you can and keep it cool during this transfer period, considerable damage has occurred to it, so that the best function we obtained in the human in any of the cadaver kidney transplants was approximately only 25 per cent of normal. I think this is a major problem at present in the use of cadaver material.

One can obtain a kidney from a person who is being guillotined or otherwise disposed of hurriedly, as perhaps the French have done. Executions do not occur too often in many states in the United States. Furthermore most of these ceremonies are scheduled, so that one can have the patient ready to receive the kidney, simply placing it in an ice bath between the time when the person donating the kidney dies and the time when one takes it to the hospital and transplants it in the recipient. Therefore I do not think one needs any elaborate method or means of preserving the kidney during this period.

I think, therefore, that the usefulness of preserved kidney homografts is relatively limited; in most experiences with homografted kidneys, the surgeon will need to obtain a living donor who can actually be scheduled to donate the kidney at the same time the patient receives it.

COMMENT: Can this suturing instrument be used on diseased vessels such as those seen in arteriosclerosis, or on dilated vessels?

ROGERS: Yes, it can be used on diseased vessels. Lapchinsky emphasizes that Androsov has often used this instrument in substituting autologous and homologous vessel segments for portions of seriously diseased, arteriosclerotic vessels in the human.

Abel A. Lazzarini, Jr. (*New York University School of Medicine, New York, N. Y.*): I was very interested in Lapchinsky's results. At New York University for the last three years we have studied the perfusion of kidneys and, on the basis of studies of organ perfusion *in vitro*, we decided to use, instead of whole blood, a preparation consisting of amino acids, antibiotics, and dialyzed protein that might preserve kidneys for some time prior to transplantation.

We use a simple family refrigerator, as Lapchinsky does, for cooling. It has a heater for warming up the organ just before reimplantation. The organ is maintained in a dialyzed membrane. We learned, as have other investigators, that one should not use a combination of oxygen and CO_2, but rather CO_2 and air. We found this rather important in the maintenance of the viability of kidney cells. These were studied in tissue culture by biopsies of the kidneys obtained at different intervals.

One interesting phenomenon concerning which I desire to inquire is that in our findings there were increases in peripheral resistance within the kidney in the first twelve hours. As you know, Murray and his group also have reported that there has been a diminution of the renal flow in the first few hours of perfusion.

We felt, therefore, that when blood is perfused for long periods of time it librates 5-hydroxytryptamine on the basis of destruction of platelets, and that this will have an effect within the organ that produces vasoconstriction.

This is of interest because Lapchinsky seems to have discussed a toxic effect; I wonder whether he has any information about the chemical components of this toxic material.

We found, interestingly enough, that if some of the 21 kidneys we perfused were preserved for more than 36 hours, there was an evident diminution of the amount of plasma cells present when they were transplanted to a homologous host previously nephrectomized bilaterally. As you know, Simonsen and Dempster have reported that it is possible that the plasma cell reaction is a graft versus host reaction. We think that by perfusion *in vitro* with our method the absence of plasma cells may imply a diminution of the graft versus host reaction.

Rogers: To try to answer that last question: one of the doctors in the Institute took perfused fluid from the leg of an animal that was undergoing a severe toxic reaction and passively transferred it to a healthy animal; the latter animal thereby suffered the same apparent toxic reaction but, interestingly, as he continued to administer the "toxic" perfusate fluid over a period of time, the healthy animal was able to pull itself out of the passively transferred toxic state. Some animals receiving the toxic perfusate died as a result of the perfusion.

As far as the chemical constituents of this fluid are concerned, Lapchinsky does not as yet know their composition, but he knows that the fluid has a high histamine content.

TOTAL BODY IRRADIATION IN MAN: TISSUE PATTERNS OBSERVED IN ATTEMPTS TO INCREASE THE RECEPTIVITY OF RENAL HOMOGRAFTS*

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The rationale for the use of total body irradiation as a preliminary to tissue transplantation relates to the ability of ionizing radiation to alter normal antibody responses. In 1908 Benjamin and Sluka[1] described a dose-dependent suppression of circulating antibodies in rabbits when beef antigen was administered within a finite time interval after irradiation, but found no effect when the appearance of antibody preceded the X-ray treatment. The literature confirming and extending these earliest observations to the definition of the radiosensitive and radioresistant phases of immune responses was reviewed by Taliaferro[2] in 1957. Thus far, data published on the impact of radiation on classic immune mechanisms in man[3] indicate that the anamnestic response follows the refractory patterns observed in certain experiments on animals.[4]

With respect to the transplanatation of homologous tissue, available evidence suggests that primary responses also may require large doses of irradiation for their effective modification. Successful bone marrow homografts appear to be the best insurance against radiation death in a variety of laboratory animals, and the chances of obtaining a permanent marrow "take" increase with increasing X-ray dosage, within the range between the marrow-effective and the supralethal thresholds.[5-10] As a corollary, the best assurance of the acceptance of fixed homologous tissue in adult populations appears to be the previous or concomitant acceptance of a bone marrow homograft from the same (or isologous) donor.[11-14]

These implications that X-ray dosage levels of potentially lethal magnitude are necessary resulted in a reluctance to apply the principle as an elective technique to humans with advanced renal disease. It was not, therefore, until 2 patients, each having lost a solitary kidney, presented themselves that it was considered justifiable to undertake the approach described below.

The first patient was a 31-year-old white female (G.L. P.B.B.H. No. 3M330) from whom a ruptured, solitary, ectopic, pelvic kidney had been removed surgically. Following 3 hemodialyses with the artificial kidney, the patient was given total-body irradiation from a 2 Mev source of the resonance-transformer type,† having a half-value layer of 7.3 mm. of lead, to a nominal mid-

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† Irradiation therapy carried out at the New England Deaconess Hospital, Boston, Mass., under the supervision of Joseph H. Marks.

plane air dose of 600 r in 3½ hours. The dose was evenly divided between anterior and posterior portals at a treatment distance of 19 feet.

During the first 48 hours after irradiation the patient was given 36 billion marrow cells, pooled from 11 indifferent donors, according to the technique described by Wilson.[15] On the fourth postirradiation day a renal homograft from a 4-year-old female was placed in the right thigh, during which procedure she received an additional 170 million nucleated marrow cells from the kidney donor. Three days later she was given 60 billion marrow cells from a male cadaver and, on the following day, 10 billion cells from 5 donors, bringing the total marrow dose to approximately 107 billion cells pooled from 16 live donors and 1 cadaver. The pertinent clinical events and hematological data are shown graphically in FIGURE 1.

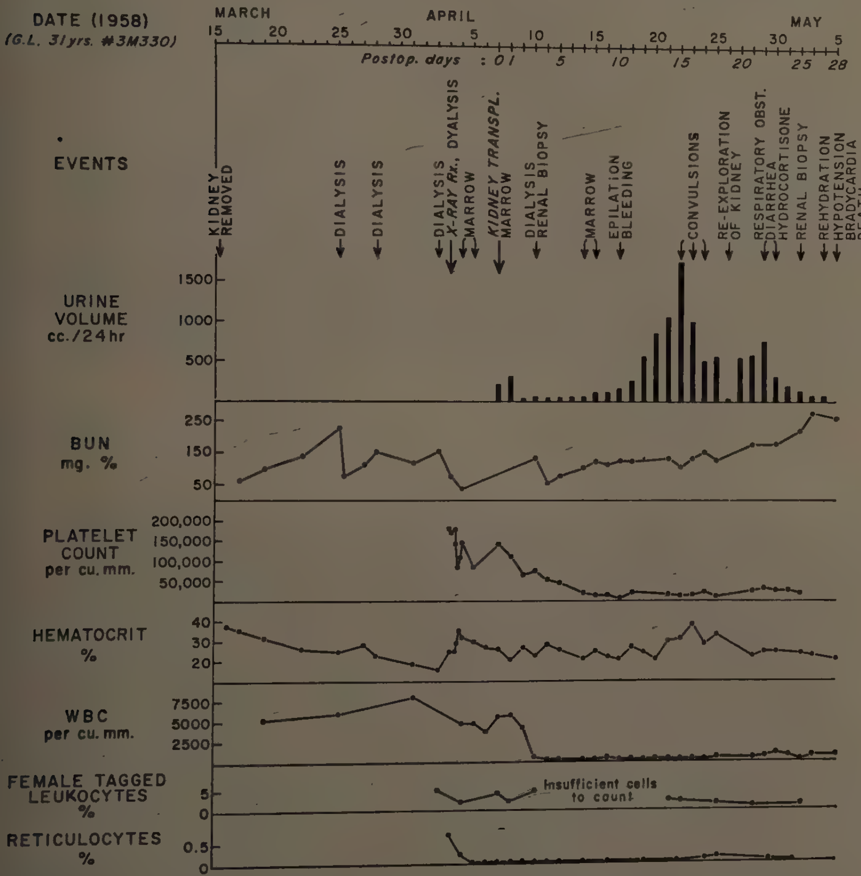

FIGURE 1. Clinical course of patient G.L. following total-body irradiation (600 r), infusion of pooled marrow, and renal homograft. Reproduced by permission from *Surgery.*

The acute-radiation syndrome was characterized by abrupt declines in the reticulocytes at 48 hours and in the total white blood count at 1 week. The

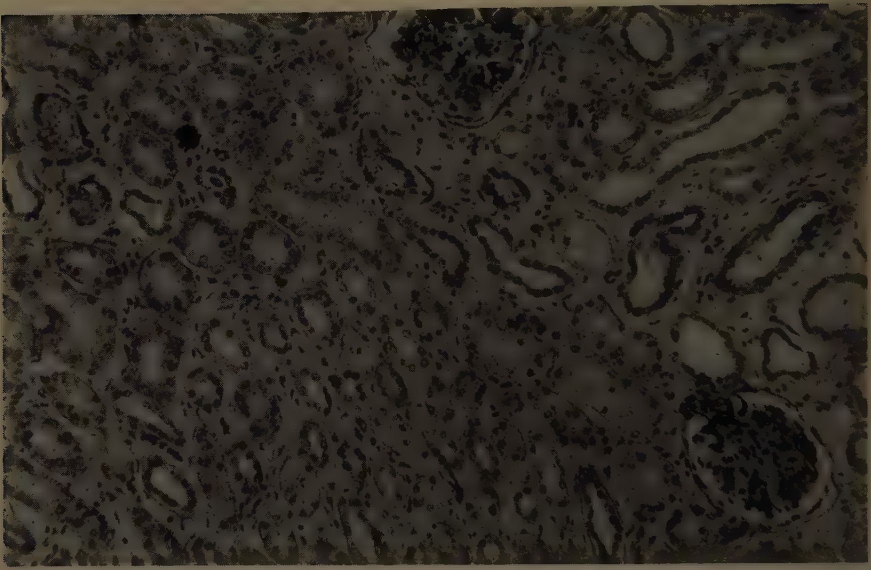

FIGURE 2. Kidney (patient G.L.). There is no evidence of a rejection pattern on day 28 after transplantation. Interstitial edema is the only abnormality. There is no cellular infiltration and no atrophy of the tubules. Hematoxylin and eosin stain. Reproduced by permission from *Surgery*.

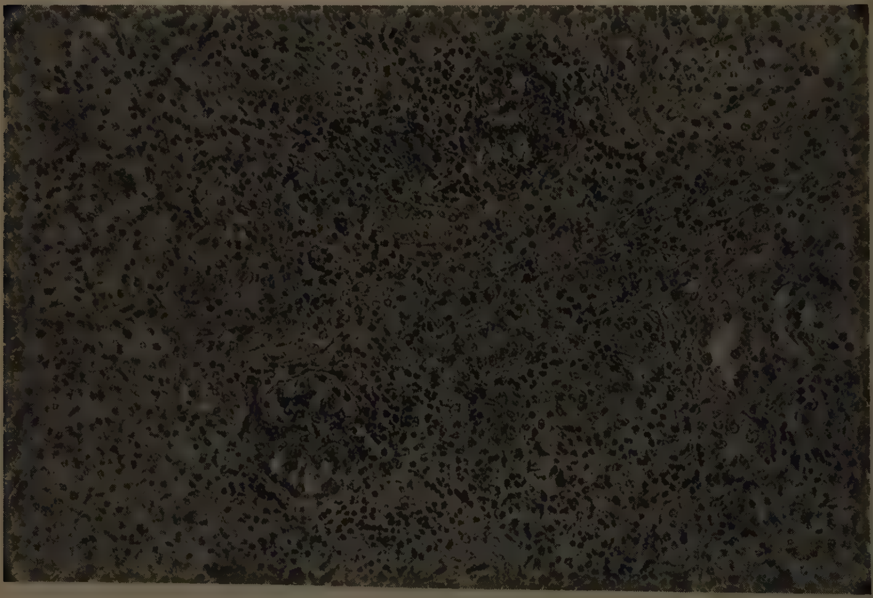

FIGURE 3. Kidney (patient D.B.). Biopsy on day 9 posttransplantation shows cellular infiltration, and the lumens of most of the tubules are unapparent. These changes represent an early rejection pattern. Hematoxylin and eosin stain.

platelets fell more slowly over 10 days to levels below the 20,000 range, these levels being largely uninfluenced by platelet transfusions every 48 hours. The thrombocytopenia was accompanied by persistent bleeding from the mouth and nasopharynx. Epilation began about 2 weeks after irradiation and progressed thereafter. The patient died 32 days after X-irradiation, with both infection and diffuse hemorrhage as prominent contributory causes. Throughout the postirradiation course she was maintained in an isolation unit under sterile precautions. Wound healing progressed in apparently normal fashion.

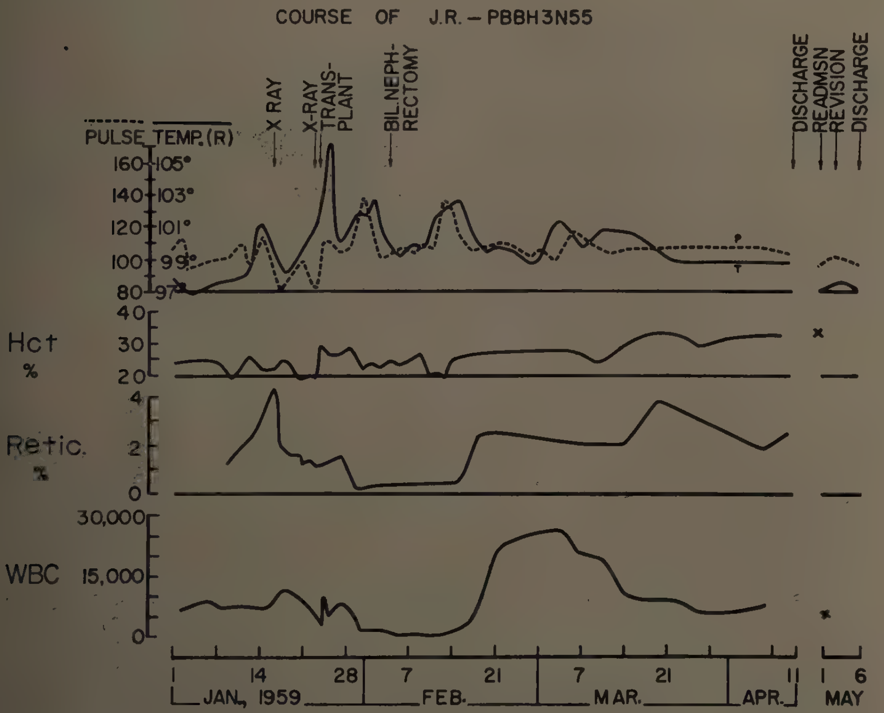

FIGURE 4. Clinical course of patient J.R. following total-body irradiation (450 r) and renal homograft.

FIGURE 2 shows the kidney as it appeared at autopsy. Except for edema, it is essentially normal, with no evidence of a rejection pattern. This is believed to be a significant observation since, in the unmodified human recipient, unmistakable evidences of rejection have been observed as early as the ninth day after homotransplantation (FIGURE 3).

The inference may be drawn that 600 r of total-body irradiation, expressed as a nominal midplane air dose, was sufficient to forestall the onset of renal homograft rejection in this patient with renal insufficiency of relatively recent origin. The dose did not permit of a successful pooled, largely indifferent, marrow homograft; indeed, the reappearance of reticulocytes in the peripheral blood shortly before death and the presence of female-tagged leu-

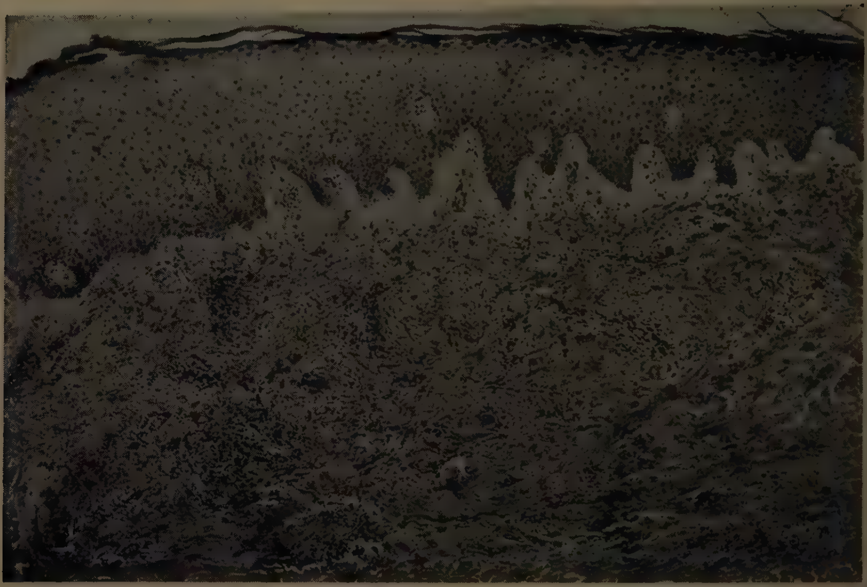

FIGURE 5. Skin (patient J.R.). The epidermis is normal, and there are scattered foci of cellular infiltration of the dermis. This biopsy was taken during the seventh month after transplantation. Hematoxylin and eosin stain.

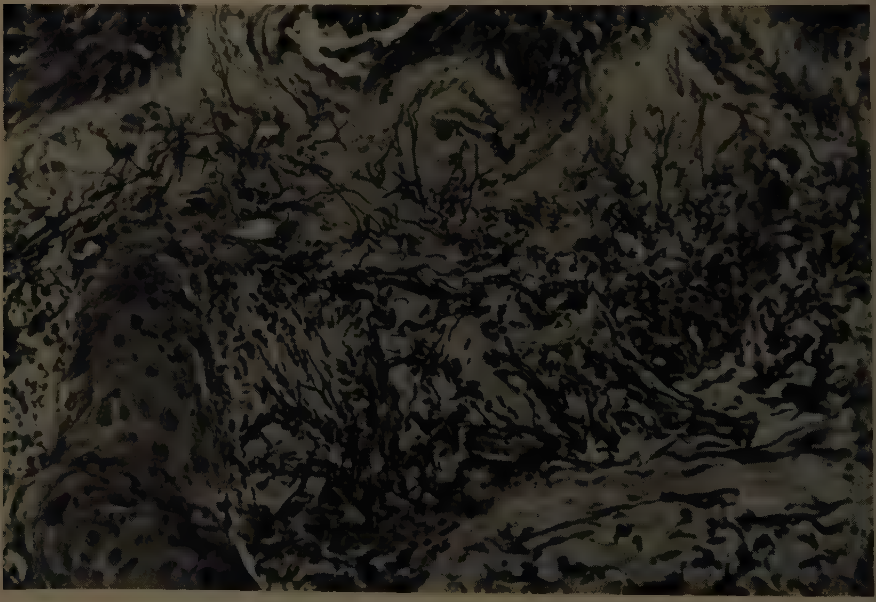

FIGURE 6. Skin. Same biopsy as in FIGURE 5, but stained for elastic fibers. The elastic fiber pattern, with fine fibrils extending to the basal epidermal layer, is normal. Further evidence for prolonged survival is the presence in the lower left field of a portion of a hair follicle. Elastic tissue stain.

kocytes in the marrow at autopsy indicate that the recipient's own marrow was recovering. The severity of the acute radiation syndrome produced by this dose of X ray, in the absence of bone marrow take, resulted in insoluble problems of sepsis and hemorrhage.

The second patient treated in the high-dose range was a 12-year-old boy (N.W. P.B.B.H. No. 3M994) whose only kidney had been sacrificed following rupture from blunt trauma. Because of the marrow failure in the first patient, a slightly higher dose of 700 r, administered by the same techniques, was delivered. At 24 hours and at 1 week following irradiation he received, respectively, 7.4 and 3.1 billion marrow cells from his mother, as the anticipated kidney donor. In the absence of any conclusive evidence of a successful marrow homograft and because of the progressive deterioration of his condition, of which bleeding was the outstanding feature, it was deemed inadvisable to sacrifice one of the mother's kidneys. The patient died 25 days after irradiation and, at autopsy, he showed extensive ulceration throughout the gastrointestinal tract. The maternal skin graft, which had been in place for only 15 days, was still intact.

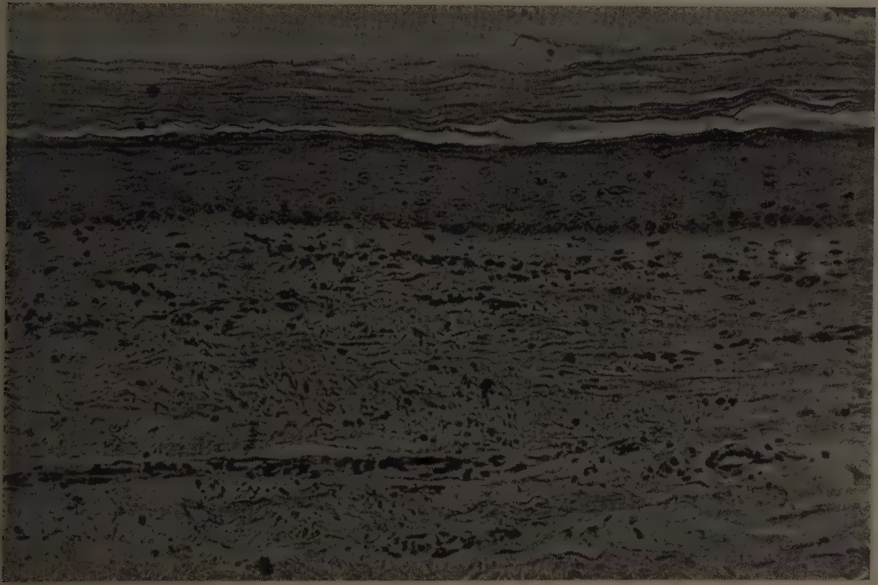

FIGURE 7. Skin (patient J.R.). Biopsy of graft 10 months posttransplantation. The epidermis is atrophic, and no accessory structures are present. Hematoxylin and eosin stain.

The conspicuous failure of these 2 pilot applications permitted only the alternatives of proceeding to even higher X-ray dose levels or attempting lower doses on a protraction schedule. Although theoretically tempting, the former method was felt to be too hazardous in its uncertainties. The impetus toward the adoption of the latter procedure was provided by the presentation of the 23-year-old preterminal chronic nephritic (J.R. P.B.B.H. No. 3N55), whose potential kidney donor was a fraternal twin with 26 identical blood groups. If quantitative gradations play a part in radiation-induced histocompatibility, the combination of the chronically uremic recipient and close consanguinity between donor and recipient offered the optimal background for evaluating the effect of irradiation doses from which recovery could be expected without marrow support.

Accordingly, 450 r were given in 2 doses of 250 r and 200 r 7 days apart. Treatment was administered, at an average dose rate of approximately 5.5 r/min., from a 250-KVP constant-potential source having a half-value layer of 3.0 mm. copper, at a target skin distance of 158 cm. The donor kidney was placed in the right lower abdomen, extraperitoneally, 24 hours after the second X-ray exposure. The clinical course of the patient's first hospitalization is charted in FIGURE 4.

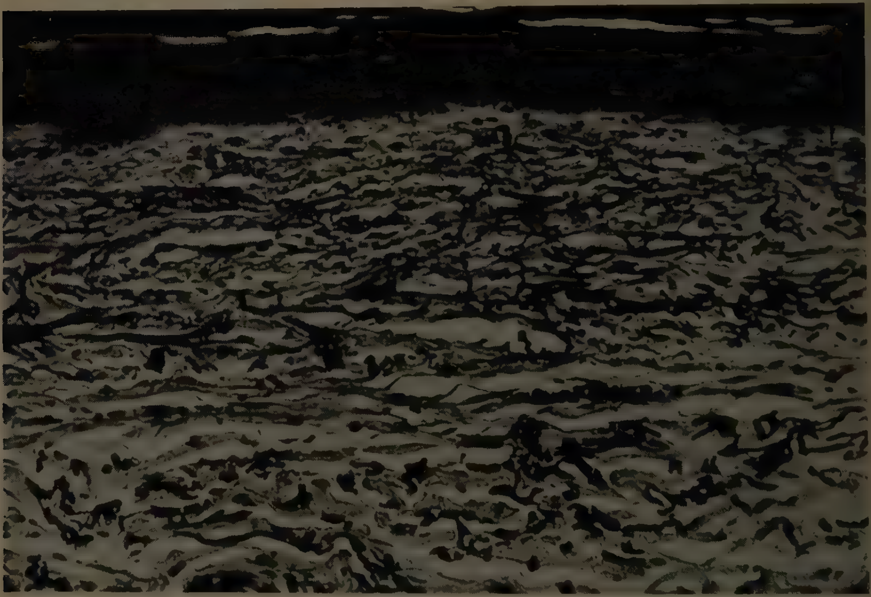

FIGURE 8. Skin. Same biopsy as FIGURE 7, but stained for elastic fibers. On the upper dermis, the elastic fibers are either absent or lacking the normal orientation to the basal epidermal layer. Elastic tissue stain.

The renal homograft functioned immediately, with an early postoperative diuretic phase that required careful control of fluid intake and electrolyte replacement. The blood urea nitrogen and blood pressure promptly returned to normal. On the eleventh postoperative day a large perinephric abscess in the right flank was drained, and both of the patient's own kidneys were removed.

Hematologically, the reticulocytes declined from the base-line level of 4.4 to 2.2 in the first 24 hours after the initial X-ray exposure; thereafter it fell slowly to a low of 0.2 at 2 weeks. Except for an early relative lymphopenia, the white blood count remained within normal ranges until the end of the second week following the first dose of X ray, when a precipitous decline took place concurrent with 2 successive daily doses of 100 r (tissue dose), each to the spleen. The platelets, also within normal ranges up to this time, underwent a comparable brisk decline to a low value of 55,000. During the third week the white count hovered at virtually uncountable levels before rising at first slowly and then sharply to rebound values in the 20,000 range by the fifth week, with many immature forms and toxic granulation. Thereafter, the course was one of slow return to and stabilization of all formed elements at normal values.

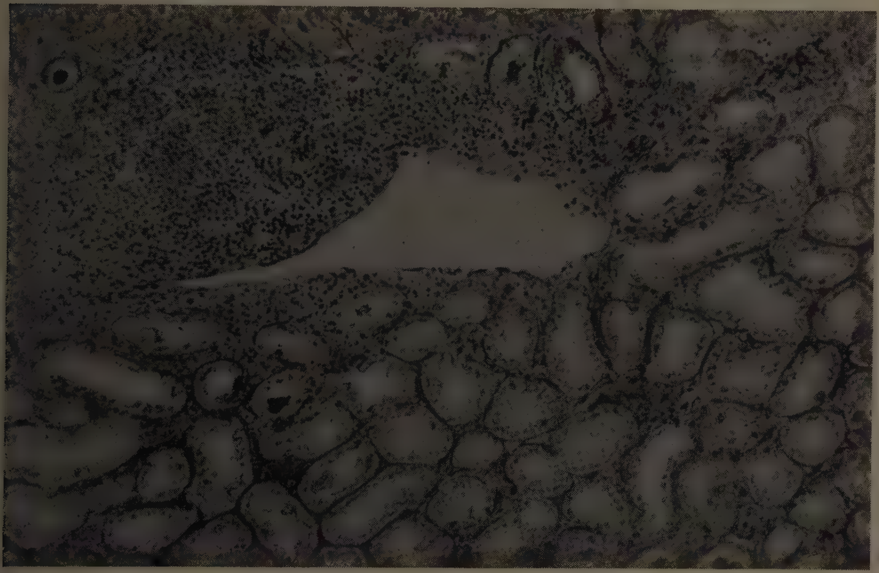

FIGURE 9. Kidney (patient J.R.). Biopsy taken at time of biopsy of skin graft (FIGURES 7 and 8). The epithelium of the tubules is normal, and they are patent. One of the scattered foci of cellular infiltration is in the upper left corner. Hematoxylin and eosin stain.

During the period of profound leukopenia the only other evidence of infection was an ulcerative stomatitis, unaccompanied by cervical adenopathy, which responded to symptomatic therapy as the white count rose. When this stormy period passed, the clinical course was one of progressive improvement, and the patient was discharged from the hospital approximately 4 months after admission.

The patient remained entirely normal and well until 6 months after transplantation, when the skin graft from his donor twin (placed prior to X ray) showed the first suspicious clinical manifestations of chronic rejection. A cellular infiltration of the dermis was present on a biopsy taken at this time (FIGURES 5 and 6), but more advanced features of a rejection pattern were lacking. The proteinuria and microscopic hematuria that were present on admission to the hospital disappeared during the period of observation.

A second skin biopsy (FIGURES 7 and 8), carried out 9 months after transplantation, showed characteristic rejection changes of loss of rete pegs and accessory structures and lack of orientation of the elastica to the basal epidermal layers. Although the patient remained clinically well and maintained normal urinary function, a renal biopsy was also done (FIGURES 9 and 10) because of persistent proteinuria, granular casts, and microscopic hematuria. Over-all renal architecture appeared quite normal, with intact glomeruli and no evidence of the patient's primary disease. There was no tubular atrophy, but cellular infiltration, spotty in distribution, was interpreted as possible evidence of the initial phase of rejection. Cellular infiltration does not occur following transplantation of an isologous kidney.

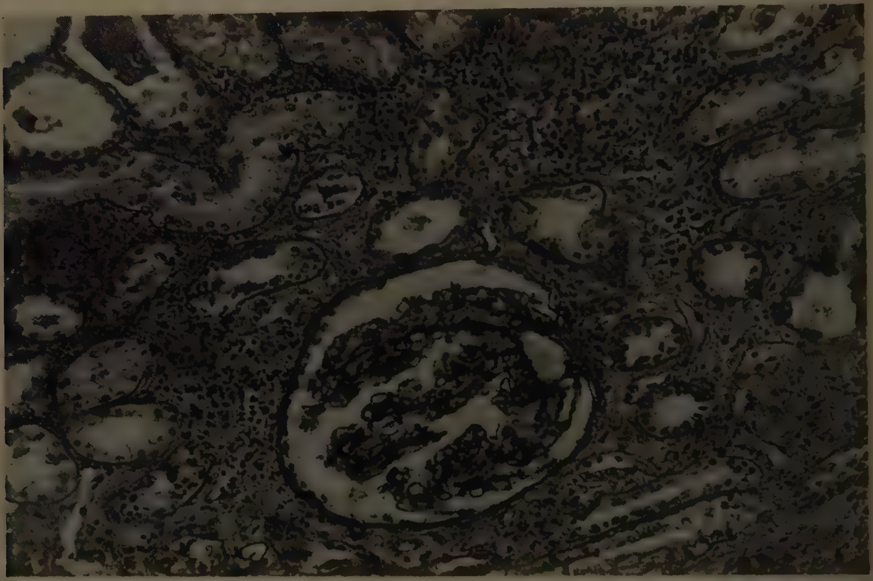

FIGURE 10. Kidney. Biopsy as in FIGURE 9 stained by periodic acid-Schiff reagent method. The tubules are normal, and there is no evidence of a primary glomerular lesion. There are scattered mononuclear cells in the interstitial tissue.

The patient was placed on prednisolone and started on a course of titrated total-body irradiation in midplane air doses of 50 r at weekly intervals. It became necessary to abandon this program after a cumulative dose of 200 r in 1 month's time because of a decline in the white count and platelets, which had been normal since discharge from his original hospitalization, to levels of 2100 and 55,000, respectively. At this writing, 1 year after transplantation, the patient's hemogram, kidney function, and urinalysis are normal except for a trace of protein and a rare red blood cell in the spun sediment.

From the standpoint of response to total body irradiation in the presumed sublethal range, one additional patient experience warrants mention. The patient, a 15-year-old subacute nephritic (M.C. P.B.B.H. No. 7N275) was given an initial dose of 250 r but, because of a drop in the white count from 7600 to 3500, the second exposure was reduced to 150 r. Although the subsequent hematological pattern was essentially the same as in the foregoing patient, bleeding and infection played overbearing roles in bringing about her demise 3 weeks after irradiation. Although the strict and elaborate precautions accorded her were shown to be effective, the patient's sepsis was on the basis of her own Gram-negative bacterial flora. A renal homograft from her father functioned well until obstructed by a blood clot and showed no evidence of an early rejection pattern at post-mortem examination.

From this limited human experience, the following conclusions may be drawn:

(1). Total body irradiation in the potentially lethal range of 600 to 700 r, administered in a single dose, provides no assurance of the acceptance of homologous bone marrow, either pooled or from a single donor.

(2) In the absence of a successful marrow homograft, radiation doses of this magnitude may lead to insurmountable problems of hemorrhage and infection. Whereas environmental sepsis can be controlled, its regulation does not influence autoinfection.

(3) Radiation doses of the order of 600 to 700 r are capable of preventing the rejection of fixed tissue homografts for at least 28 days.

(4) Total-body irradiation in the range of 400 to 450 r, in divided doses at weekly intervals, results in varying patterns of severity of the acute radiation syndrome.

(5) Sublethal total-body irradiation may delay the rejection of fixed tissue homografts by the chronically or subacutely uremic recipient. Under circumstances of close consanguinity such delay may be of significant time dimensions.

References

1. Benjamin, E. & E. Sluka. 1908. Antikörperbildung nach experimenteller Schädigung des hämatopoietischen Systems durch Röntgenstrahlen. Wien. klin. Wochschr. **21:** 311.
2. Taliaferro, W. H. 1957. Modification of immune response by radiation and cortisone. Ann. N. Y. Acad. Sci. **69**(5)**:** 745.
3. Stoloff, I. L., F. I. Haurani, E. F. Repplinger & W. P. Havens, Jr. 1959. Effects of total-body irradiation on the production of antibody in man. New Engl. J. Med. **260:** 1258.
4. Dixon, F. J., D. W. Talmage & P. H. Maurer. 1952. Radiosensitive and radioresistant phases in antibody production. J. Immunol. **68:** 693.
5. Lorenz, E., D. Uphoff, T. R. Reid & E. Shelton. 1951. Modifications of irradiation injury in mice and guinea pigs by bone marrow injections. J. Natl. Cancer Inst. **12:** 197.
6. Congdon, C. C. & I. S. Urso. 1957. Homologous bone marrow in the treatment of radiation injury in mice. Am. J. Pathol. **33:** 749.
7. Porter, K. A. 1957. Effect of homologous bone marrow injections in X-irradiated rabbits. Brit. J. Exptl. Pathol. **38:** 401.
8. Fishler, M. C., L. J. Cole, V. P. Bond & W. E. Milne. 1954. Therapeutic effect of rat bone marrow injection in rats exposed to lethal whole body X-radiation. Am. J. Physiol. **177:** 236.
9. Rekers, P. E., M. P. Coulter & S. Warren. 1950. Effect of transplantation of bone marrow into irradiated animals. Arch. Surg. **60:** 635.
10. Ferrebee, J. W., H. L. Lochte, Jr., A. Jaretski, III, O. D. Sahler & E. D. Thomas. 1958. Successful marrow homograft in the dog after irradiation. J. Surg. **43:** 516.
11. Main, J. M. & R. T. Prehn. 1955. Successful skin homografts after administration of high dosage X-radiation and homologous bone marrow. J. Natl. Cancer Inst **15:** 1023.

12. Trentin, J. J. 1956. Mortality and skin transplantability in X-irradiated mice receiving isologous, homologous, or heterologous bone marrow. Proc. Soc. Exptl. Biol. Med. **92:** 688.
13. Trentin, J. J. 1956. Effect of X-ray dose on mortality and skin transplantability in mice receiving F_1 hybrid marrow. Proc. Soc. Exptl. Biol. Med. **93:** 98.
14. Wilson, R. E., J. B. Dealy, Jr., N. L. Sadowsky, J. M. Corson & J. E. Murray. 1959. Transplantation of homologous bone marrow and skin from common multiple donors following total body irradiation. J. Surg. **46:** 261.
15. Wilson, R. E. 1959. Technics of human-bone-marrow procurement by aspiration from living donors. New Engl. J. Med. **261:** 3.

Discussion of the Paper

Delta Uphoff (*National Cancer Institute, Public Health Service, Bethesda, Md.*): How much, if any, did the irradiation in these cases help? Second, how much benefit was afforded by the uremic condition of these patients? Finally, was there any synergistic effect afforded by the irradiation and the uremia? I know of no animal work that would lead to possible answers to these questions.

Superimposed upon this, we have the academic problem of whether there could have been a matching of histocompatibility genes in these twins. I think the answers to these questions will remain unanswered for some time.

The disadvantage of using very high doses of irradiation in man make it advisable to re-evaluate the irradiation-dose problem in experimental animals. Previous work on mice has indicated that a dose range of 500 to 550 r at approximately an LD_{15}, despite inoculation of homologous marrow, would result in the death of the treated mice.

Recent work that is still incomplete indicates that this is true only in a limited number of strain combinations. In some cases, a lower dose of irradiation plus homologous marrow results in an increase in the long-term survival over the same genetic combination exposed to the LD_{100} dose level.

It would be premature to speculate on the applicability of these observations to clinical problems at this time, since tests on the chimeric state of these animals are still incomplete.

Suffice it to say that at this time the work of Main and Prehn still holds, that at the lower inoculation dose levels, where there was a matching of the major histocompatibility genes, skin transplantation may be successful.

With this bit of encouragement it is hoped that more progress will be made in the very near future, so that, at the Fifth Tissue Homotransplantation Conference, answers will be available for some of the questions raised during this conference.

Joseph W. Ferrebee (*The Mary Imogene Bassett Hospital, Cooperstown, N. Y.*): I am sure the clinicians are adjusted to the notion that fitting any scheme of treatment to an individual patient is often quite complicated. The best thing about the treatment, transplantation, and subsequent course of this patient of Murray and of his colleagues, is that while these physicians may be somewhat confused regarding everything that happened, the patient recovered and went home.

This, I think, is pretty good considering our lack of knowledge of the variables discussed in this monograph. It should not discourage our trying to fit this scheme of therapy to other patients.

The problem of working a similar scheme at higher doses of irradiation is complicated by the fact that in man one does not know how much radiation one needs to achieve a good bone marrow transplant. I do not believe that anyone has had a truly good bone marrow transplant in a human patient following radiation exposure. There have been numerous instances of the temporary activity of marrow grafts after irradiation exposures.

For the type of problem that Murray and Merrill have, this is perhaps not good enough. It is evident, however, that in large animals such as the dog and man, the amount of irradiation required to get a good marrow transplant is considerably greater than that which we have learned to expect is necessary in small rodents. I think that Thomas and others have some rather encouraging evidence in this direction.

Dogs will tolerate as much as 1800 or 2000 r of total body irradiation if they are properly prepared and taken care of and, at these dose levels, the incidence of good homografts of bone marrow seems encouraging—to me, at least. Man apparently will tolerate at least 1600 r. One of Thomas's patients has gone home with restored marrow function after 1600 r of full-body irradiation. Of course, this was done by using bone marrow taken from his twin. However, it at least indicates that 1600 r properly administered does not necessarily produce fatality from destruction of the intestine, recurrent infections, and that type of complication.

In fact, I think the intestines of an individual with whom Thomas has recently worked received 2000 r, which is encouraging. This individual did not survive but I don't believe the mortality was attributed to intestinal trouble.

Some of the dogs that have had a great deal of irradiation in marrow transplantation have died of the secondary disease syndrome, but I am not convinced that this is not a treatable syndrome.

Using one of Uphoff's suggestions, Mannick, about a year ago, gave some methotrexate to a dog at presumably the right time or, perhaps, just gave it to him, and the dog did well. However, the dog is now alive, one year after 1800 r. It is a male dog and still has female polys. It is somewhat gray, but it seems quite normal.

Recently, Thomas has given it a dose of distemper vaccine, and the titer has jumped from a zero level to 2,500. Evidently, as rodents do, it has regained its general reactivity to environmental pathogens. Moreover, it has been off medicine, antibacterial agents, and sera for about four or five months. It seemingly is quite all right.

There is another nutritional problem in the secondary disease syndrome that is quite marked in some instances. In some of our dogs, the digestion is poor. Meat fibers come through undigested; fat comes through unassimilated. At post-mortem, the pancreas in some of these animals is in a very bad condition. Since the endocrine function does not seem to be in bad condition, one presumes that this is a treatable entity that can be handled by giving oral pancreas preparations.

It think these varied experiences in this field would encourage one in selected clinical circumstances, especially for the patient's morale if perhaps for nothing else, to keep trying to employ steps in this direction, because we still know very little about how to manage radiation in its effect upon the receptivity of homografts.

MERRILL: May I emphasize the point that Dealy has made about infection, at least in the human situation. We have gone to a great deal of effort through the help of Carl Walter and his group to eliminate extraneous pathogens, and it is a great disappointment to us to realize that we are still in difficulty from the patient's own organisms. This remains one of the fundamental problems, I think, in handling both patients and animals.

I make a plea for those of you who are interested in this problem to think very carefully not only about the problem at the human level, where we have pretty good facilities for taking care of sick patients, but at the animal level, particularly at the dog level, because it seems to me a major problem in working this thing out in the dog will be the provision of facilities for taking care of a sick, irradiated dog. We don't have these facilities now, and I think it will be critically important for further progress in this field.

JOSÉ VIÑAS (*Barcelona, Spain*): May I comment on the possibility of a slight change in timing of these events in the grafting of kidneys or other organs. It seems to me that perhaps one might first transplant the kidney in the nonirradiated animal or patient, and then a few days later, before one can expect the immunological responses to the graft, apply the irradiation. We shall find at this point the immunologically competent cells from both the graft and the host at a particularly susceptible stage to the effects of X-ray therapy. Subsequently, one could then infuse a large amount of lymphoid cells, perhaps obtained from fetal liver and spleen, in suspensions that would permit the host to become tolerant to graft antigens. Using these methods, we might slightly improve the survival time of the grafts.

Another point I propose to make is that perhaps we should look for means other than X rays to destroy the reactivity of the lymphoid tissue and other immunologically active tissue. One of these perhaps could be a substance referred to in a recent paper in *Nature*, which is apparently an enzyme extracted from soya beans that can be injected into animals either intraperitoneally or intravenously or even applied locally. It reportedly has the same depressant effects on immunological tissues as those obtained by the use of X rays.

MURRAY: I am sure that the search for a chemotherapeutic substitute for X rays in such problems as these is diligently under way at many centers, and may prove immensely valuable in difficult clinical conditions, as described by Dealy.

DEALY.: To answer Viñas' comments, of course I agree that the time of the actual presentation or release of the antigen is supposedly the irradiation-sensitive time insofar as antigen-antibody responses of the primary type are concerned, but I think that to approach this problem in a methodical way we must wait until we are able to demonstrate antibodies to tissue antigens, to be definitely sure whether we are more correct in giving the X rays, prior to transplantation or whether we should reverse the experimental protocol.

Again, I certainly agree with Uphoff. I think that one of our problems in dealing with this particular patient who had the homograft from his fraternal twin is for us to keep his gratitude and enthusiasm from distorting our own perspective.

On the other hand, we are faced with the fact that the healthy twin rejected the skin homograft from the sick twin and, at least in my opinion, if the patient had maintained both the skin and the kidney up to this point with no evidence of rejection, then I think we should all need to say that we did not know whether the X rays had anything to do with his apparent acceptance of these tissues.

However, we have been faced with the real situation in which the subject has now lost this skin homograft. We even think he is undergoing an early renal rejection, possibly modified by subsequent irradiation, so that although it is not proof conclusive, I think that it at least affords a broad hint that irradiation conspired with other factors, such as close consanguinity and the uremic state of the recipient to favor prolonged survival and function of the kidney transplant.

THE FATE OF SULFATED MUCOPOLYSACCHARIDES OF A CORNEAL GRAFT*

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Introduction

The fundamental problem in keratoplasty lies in the changes that occur in the corneal graft. Opinions differ as to whether a corneal transplant, when taken, will survive or will be replaced, wholly or partly, in its new environment.

The elimination rate of S^{35}-sulfated mucopolysaccharides of the ground substance of corneal grafts was studied by Dohlman[1,2] after Dziewiatkowski[3,4] had shown that most of the inorganic radioactive sulfate administered was rapidly excreted, and that only a small fraction was retained and incorporated into sulfated mucopolysaccharides throughout the animal. Meyer *et al.*,[5,6] in a thorough analysis of bovine cornea, definitely established the presence of 3 compounds: chondroitin sulfate A, chondroitin, and (the largest single fraction, accounting for one half of the mucopolysaccharide content), keratosulfate. Thus the cornea, being rich in sulfated polysaccharides, reveals significant uptake of S^{35}, as demonstrated.[7] Three days after administering S^{35} it was found that 1.9 per cent of the total corneal radioactivity stemmed from inorganic sulfate and 4.5 per cent from organic nonsulfate sulfur, such as amino acids.[7] After 17 and 43 days, there was little change noted in the above data. It was observed also that the *in vivo* half life of incorporated S^{35} in rabbit cornea was 32 days. It was claimed, however, by Nathaniel and Levene,[8] that the *in vivo* half life was considerably shorter, that is, 8 days.

Quantitative studies of the elimination rate of S^{35}-sulfated mucopolysaccharides of homografts were performed by Dohlman,[1] who demonstrated that in freshly transplanted homografts radioactivity decreased to approximately two thirds after 2 weeks and thereafter resumed its normal rate of elimination. In another study[2] S^{35} uptake into bound organic substances was determined *in vitro* quantitatively. It was found that the S^{35} uptake by the grafts was greatly reduced immediately after surgery and did not reach normal values until at least 10 days later. It was therefore assumed that a postoperative net loss of mucopolysaccharide sulfate occurred after surgery and that the interfibrillar ground substance was rapidly substituted by the host.

In the investigation reported here we attempted to determine the route of S^{35} elimination and whether the S^{35}-elimination rate of labeled donor tissue depends upon its viability. In addition, quantitative and qualitative procedures were employed to determine whether sulfated mucopolysaccharides of the host entered the donor cornea. In the above study 4 groups were used:

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(1) fresh donor tissue, (2) cornea treated with formalin, then transplanted, (3) cornea preserved in 10 per cent aqueous polyvinylpyrrolidone (PVP K-60), stored for 12 days and then transplanted and, finally (4) untreated cornea stored for 12 days prior to transplantation. The interest taken in PVP-preserved tissue was based on experiments, thus far unpublished, indicating that, with the use of PVP as a corneal preservative, the structural integrity and state of hydration of cornea is maintained for a longer period of time than nonpreserved stored cornea. Pilot studies[9] of corneal penetrating grafts stored for 7 days gave successful clinical results. Paton, in a discussion of the presentation of Stocker *et al.*,[10] concluded that successful grafts may be obtained after prolonged storage with polyvinyl plastic, but that they are clinically less suitable than fresh cornea. Experiments currently performed indicated that rabbit cornea could be preserved for as long as 12 days and still yield successful clinical results.

Method

Male New Zealand albino rabbits weighing 1.8 and 2.2 kg. were used. They were allowed food (Wayne Rabbit Pellets) and water without restriction. Donor and host animals were treated before surgery as follows: each received intravenously at the marginal ear vein 1.0 mc. carrierfree S^{35}-labeled sodium sulfate (pH7) per kilogram of body weight. Three days later, when most of the inorganic S^{35} had been eliminated, the animals were used.[7]

Controls. Fifteen rabbits (21 corneas used) were treated as above and sacrificed. From each cornea, a 5-mm. center disk was trephined out, and with a 10-mm. trephine the cornea was reduced to a peripheral ledge or ring. A quantitative study of S^{35}-incorporated material of both disk and ring was made after each was dried, as described below.

Hot donor-cold host. Three days after the administration of the isotope, the animals were sacrificed with Nembutal. Their corneas were then divided into 4 groups: (1) fresh, (2) formalin-treated, (3) 12 P, and (4) 12 NP, respectively prepared as follows:

(1) *Fresh.* Five-mm. disks were removed from each eye and 1 was immediately transplanted to an unlabeled animal; the other was stored at −15° C. until the host animal was sacrificed for comparison.

(2) *Formalin-treated.* Five-mm. disks were removed from each eye. One was stored at −15° C., the other was treated with 10 per cent neutral formalin for 5 min. and then suspended in isotonic saline for 30 to 45 min. prior to transplantation to an unlabeled rabbit.

(3) *12 P.* Animal sacrificed and eyes enucleated. Eyes then were washed for 5 min. in polymyxin B (Neosporin*). Employing sterile technique, the eyes were held in gauze, and the aqueous humor was aspirated with a 26-gauge needle and tuberculin syringe after entry was made through the limbus into the anterior chamber. After the aqueous humor had been aspirated the chambers were filled with 10 per cent aqueous sterilized solution of polyvinylpyrrolidone (PVP K-60), using approximately 0.3 cc. more volume than was initially aspirated. The eyes were then washed again for 2 min. in Neosporin and

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stored for 12 days at 5° C. in a sterile ground-glass-stoppered bottle upon a dental cotton ring saturated with 10 per cent aqueous K-60 PVP. After 12 days 5-mm. disks were removed from those eyes; one disk was transplanted to a cold animal and the other was stored at −15° C.

(4) *12 NP.* The eyes were enucleated, washed, and stored as above for 12 days. After 12 days of storage, one 5-mm. disk from 1 eye was transplanted to a host, while its mate was stored at −15° C. until the host animal was sacrificed.

The labeled grafts in each group were transplanted to the corneas of unlabeled rabbits. Both eyes of the unlabeled rabbits were operated upon within a period of 1½ hours. Of every 2 unlabeled rabbits operated upon, 1 would have transplanted to its corneas a labeled fresh transplant to 1 eye and a formalin-pretreated cornea transplanted to the other. The other rabbits would have 12 P transplanted to one eye and 12 NP transplanted to the other. After 7 or 14 days the animals were sacrificed with Nembutal, the eyes were examined carefully, and the clinical state of each was recorded. Surgical failures, infections, massive synechiae, or partially sloughed grafts were discarded. The grafts were removed gently from the host cornea after any remaining sutures were cut and discarded. The grafts and their mates, which were stored initially at −15° C., were dried at 100° C. over phosphorous pentoxide under vacuum for from 2½ to 3 hours or to constant weight. Five quantitative determinations were made for each group and its mates after 7 days, and 5 for each group and its mates after 14 days. A total of 40 corneal grafts and 40 mates was used for quantitation. Two grafts for each group of 7-day animals and of 14-day animals were used for radioautographs. A total of 16 grafted eyes was used for radioautography and histology.

Cold donor-hot host. The identical procedure was followed as above for preparation and surgery of fresh, formalin-treated, 12 P, and 12 NP corneas. However, this experiment involved the transplantation of non-S^{35}-radioactive 5-mm. donor disk (fresh, formalin-treated 12 P or 12 NP) to a rabbit treated 3 days previously with isotope. Of every 2 labeled rabbits 1 fresh disk was transplanted to 1 eye and a formalin-treated disk to the other. The other animal would have a 5-mm. 12 P disk transplanted to 1 eye and a 5-mm. 12 NP disk to the other. The animals were sacrificed 7 or 14 days after surgery. After surgical and other failures were discarded, the grafts were removed as described above. Seven days after surgery 5 quantitative determinations were made for 12 P and 5 for 12 NP, as well as 8 for each fresh and formalin-treated cornea on the fourteenth day after surgery. A total of 46 corneal grafts and 46 rings was used for quantitation. In addition, for each group of 7- and 14-day animals, 1 was used for radioautographic and histological purposes—a total of 8.

Quantitative data. The procedure for S^{35} determination used by Moltke[11] was employed here. Dry and weighed tissue was placed in macro-Kjeldahl flasks. One half cc. of 12.5 per cent aqueous sodium sulfate solution and approximately 5 cc. of fuming nitric acid were added and boiled under a fume hood until dry. Approximately 1 cc. of fuming nitric acid was added again and allowed to boil to dryness. The residue was completely white upon drying. It was allowed to cool and was dissolved completely with 15 cc. distilled water to which was added 2 drops of concentrated hydrochloric acid. The mixture was then allowed to boil. To the boiling solution was added 1.5 cc. of 25 per cent aqueous barium chloride solution, and the mixture was then cooled and filtered with suction through a Tracerlab precipitation apparatus E8B and a glass-fiber filter paper* 2.1 cm. diameter No. 934-AH. After making a quantitative transfer, acetone was added and the filtrate allowed to dry. The filter paper and precipitate were gently removed and placed in a planchet. The diameter of the planchet was 2.8 cm. The precipitate was at infinite thickness for S^{35} (25 mg./cm.^{2}). A stainless steel ring with an outer diameter of 2.6 cm. and an inner diameter of 1.6 cm. was placed over the precipitate in the planchet for constant area. The samples were read in a Tracerlab glass-flow counter (1.3 per cent butane, 98.7 per cent helium), and corrections were made for background. Samples were counted for 10 min. and recounted to hold the counting error well within 5 per cent. If the sample gave less than 1000 counts/10 min., then at least 1000 counts were registered. If the counts were within 5 per cent error, then the average of the 2 was taken and recorded. Activity is expressed as either counts per minute per cornea (cpm/cornea) or counts per minute per dry weight of corneal tissue (cpm/dry wt. of tissue), as indicated.

Surgical technique. Rabbits were anesthetized with Veterinary Sodium Nembutal (60 mg./cc.) administered into the marginal ear vein. The eye was flushed with Neosporin solution, with 2 drops of 4 per cent atropine added. Two 4-0 eye-silk sutures were put through the conjunctiva and tied to a metal disk that fitted around the operative area to stabilize the eye during surgery. Retrobulbar injection of 2 per cent lidocaine (Xylocaine) hydrochloride was given at 6 o'clock and at 12 o'clock. A 5-mm. round penetrating graft was cut out completely with a Katzin trephine, and the cut was completed with curved corneal scissors. The donor eye was similarly treated. The cornea was transplanted and 6-0 chromic surgical gut sutures (Ethicone No. 790), with attachment minimal trauma needles, were used. Eight interrupted sutures were evenly spaced about the cornea. From time to time during surgery a few drops of heparin sodium (1000 U.) were used to minimize fibrin formation. After surgery and on the following day a few drops of 4 per cent atropine and polymyxin B-bacitracin (Polysporin) antibiotic ointment were administered. Surgery was done under sterile conditions.

Histology. Eyes were fixed in 10 per cent neutral formalin for 2 days and washed overnight and dehydrated in 70, 80, 95, and 100 per cent ethyl alcohol for 5 hours each. They were then placed in xylene until they cleared, then in paraffin for 4 to 5 hours and embedded, cut at 7 μ and then stained with hematoxylin and eosin, Alcian blue, toluidine blue, and PAS.[12]

Radioautography.[13] Tissues were fixed and cut (7 μ) as above. The ribbons were transferred to the darkroom, placed in a water bath, and floated onto a photographic slide. Kodak spectroscopic plates 1 × 3 type autoradiographic NS (2) and A were used. They were then placed in a slide box containing $CaCl_2$ at one end. Photographic black tape sealed side openings, and the slide box was placed in the refrigerator. Upon drying the sections remained permanently attached to the photographic emulsion. After proper exposure they were deparaffinized, hydrated, and developed in Kodak D-19 developer for 2 to 5 min., washed in tap water for 15 sec. and fixed for 20 min. in the darkroom.

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* Reeve Angel and Co., New York, N. Y.

TABLE 1

CONTROLS

 5-mm. Disk			 10-mm. Ring			>% S^{35} in 5-mm. Center disk
cpm	Dry wt. (mg)	cpm/Mg. dry wt.	cpm	Dry wt. (mg.)	cpm/Mg. dry wt.	
247	1.9	130	678	6.2	109	19.3
227	1.8	126	710	6.4	111	13.5
348	1.8	193	1154	6.4	180	7.2
349	1.9	184	1169	6.4	183	0.0
368	2.5	147	949	7.9	120	22.5
332	2.2	151	969	7.6	128	18.0
304	2.0	152	847	7.7	110	38.2
298	1.9	158	841	7.4	114	38.5
343	1.9	181	1111	7.5	148	21.6
294	1.9	155	1068	7.4	144	6.3
263	1.8	146	963	7.7	125	16.8
273	1.9	144	830	6.9	120	20.0
203	1.9	107	1250	14.0	89	20.2
211	2.0	106	1091	11.7	93	14.0
174	2.3	76	398	5.7	70	8.6
216	2.2	98	542	5.7	95	3.2
249	2.3	108	972	11.4	85	25.9
210	1.9	111	795	9.8	81	37.0
109	2.1	52	366	7.7	48	8.3
187	2.4	78	360	5.0	72	8.3
291	2.0	146	904	7.0	129	13.2

Average % difference: 18.0.
Smallest % difference: 00.01.
Largest % difference: 38.5.
Number of animals: 15.
Number of cornea: 21.

They were allowed to dry in the refrigerator for 2 hours. Counterstain with hematoxylin was desirable and was done to some sections.

Results

Controls. Two unlabeled animals were chosen at random and their whole corneas tested for any radioactive sulfur. In each case the cpm were the same as background.

In TABLE 1 the quantitative data of disks and rings are compared after 3 days of administering the isotope. The cpm, dry weights, and cpm/dry weight are given. It was noted that of 21 corneal 5-mm.-center disks and 10-mm. rings quantitated for S^{35} (cpm/dry wt.), all center disks yielded greater values than did the rings. The smallest percentage difference was an insignificant fraction over that of the ring (0.01 per cent), the largest being 38.5 per

TABLE 2

HOT GRAFT-COLD HOST, 7 DAYS AFTER SURGERY

Per cent S^{35} Remaining in Donor

cpm/Cornea		Per cent remaining	Clinical state	cpm/Cornea		Per cent remaining	Clinical state
Donor	Control			Donor	Control		
	Fresh				Formalin		
136	216	63.0	Opaque	119	174	68.4	Opaque
107	148	72.3	Clear	303	408	74.3	Clear
292	456	64.0	Clear	278	405	68.6	Opaque
269	405	66.4	Clear	608	921	66.0	Opaque
236	347	68.0	Clear	250	355	70.4	Opaque
Average		66.7		Average		69.5	
	12 P				12 NP		
134	211	63.5	Opaque	137	203	67.5	Opaque
345	461	74.8	Clear	305	494	61.7	Clear
195	271	72.0	Clear	361	512	70.5	Opaque
244	359	68.0	Clear	198	288	68.8	Opaque
252	397	63.4	Clear	264	432	61.1	Opaque
Average		68.2		Average		65.9	

TABLE 3

HOT GRAFT-COLD HOST, 14 DAYS AFTER SURGERY

Per cent S^{35} Remaining in Donor

cpm/Cornea		Per cent remaining	Clinical state	cpm/Cornea		Per cent remaining	Clinical state
Donor	Control			Donor	Control		
	Fresh				Formalin		
38	109	34.9	Opaque	58	187	31.0	Opaque
109	241	45.2	Clear	175	464	37.7	Opaque
158	387	40.8	Clear	210	697	30.1	Opaque
214	508	42.1	Clear	228	535	42.6	Opaque
104	260	40.0	Clear	98	261	37.5	Opaque
Average		40.6		Average		35.8	
	12 P				12 NP		
169	364	46.4	Opaque	138	407	33.9	Clear
534	1248	42.8	Clear	450	1104	40.8	Opaque
98	234	41.9	Clear	210	480	43.8	Opaque
94	259	36.3	Clear	111	325	34.2	Clear
95	250	38.0	Clear	122	337	36.2	Opaque
Average		41.1		Average		37.8	

TABLE 4

HOT HOST-COLD DONOR, 7 DAYS AFTER SURGERY

Prelabeled S^{35} Donor Uptake

cpm/Mg. dry wt.		Clinical state	cpm/Mg. dry wt.		Clinical state
Donor	Ring		Donor	Ring	
Fresh			Formalin		
147	194 + Ring	Clear	239	118 + Donor	Translucent
167	153 + Donor	Clear	264	99 + Donor	Opaque
89	75 + Ring	Translucent	50	56 + Ring	Opaque
37	62 + Ring	Clear	27	63 + Ring	Clear
30	71 + Ring	Clear	48	55 + Donor	Opaque
88.6	111	Average	125.6	78.2	
4 + Ring, 1 + Donor			3 + Donor, 2 + Ring		
12 P			12 NP		
67	98 + Ring	Translucent	132	88 + Donor	Opaque
56	90 + Ring	Translucent	78	61 + Donor	Opaque
39	78 + Ring	Clear	64	78 + Ring	Opaque
28	75 + Ring	Clear	23	72 + Donor	Clear
30	72 + Ring	Clear	74	34 + Donor	Opaque
44.0	86.0	Average	74.2	66.6	
5 + Ring, 0 + Donor			4 + Donor, 1 + Ring		

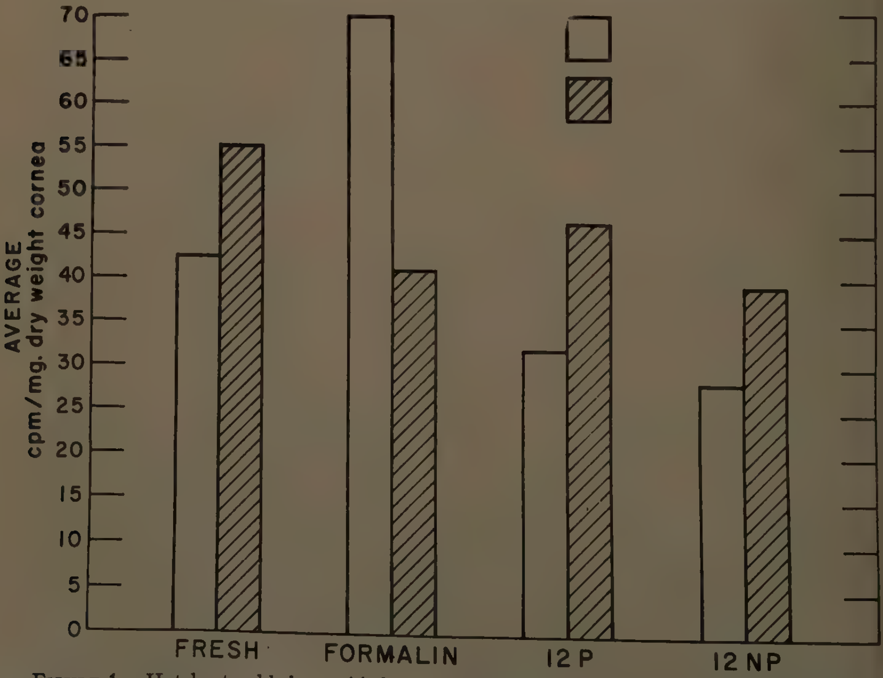

FIGURE 1. Hot host-cold donor 14 days after surgery: uptake of prelabeled S^{35}. *White bars*, donor; *shaded bars*, ring.

cent. The experimental error, including dry weights and counting was found to be ±5 per cent. The very large variation can be attributed only to the individual variation of the animals tested, as evidenced by the varying uptake of S^{35} among the animals. However, it is significant that, of 21 corneas, all except 1 indicated a higher concentration of S^{35} toward the center disk.

TABLE 5
HOT HOST-COLD DONOR, 14 DAYS AFTER SURGERY
Prelabeled S^{35} Donor Uptake

cpm/Mg. dry wt.		Clinical state	cpm/Mg. dry wt.		Clinical state
Donor	Ring		Donor	Ring	
Fresh			Formalin		
152	87 + Donor	Clear	53	31 + Donor	Opaque
29	37 + Ring	Clear	121	47 + Donor	Opaque
21	40 + Ring	Clear	105	44 + Donor	Opaque
28	58 + Ring	Translucent	59	29 + Donor	Opaque
26	53 + Ring	Clear	26	43 + Ring	Opaque
25	49 + Ring	Opaque	79	47 + Donor	Opaque
34	70 + Ring	Translucent	60	55 + Donor	Opaque
25	47 + Ring	Translucent	58	33 + Donor	Opaque
26	49	Median	59	43	
42.5	55.1	Average	70.0	41.1	
−12.6 (< Ring)		Average diff.	+28.9 (> Ring)		
7 + Ring, 1 + Donor			7 + Donor, 1 + Ring		
12 P			12 NP		
70	41 + Donor	Opaque	61	54 + Donor	Opaque
24	47 + Ring	Clear	20	35 + Ring	Opaque
27	50 + Ring	Clear	17	45 + Ring	Opaque
22	51 + Ring	Clear	22	42 + Ring	Clear
17	44 + Ring	Clear	23	52 + Ring	Opaque
22	44	Median	20	42	
32.0	46.6	Average	28.6	39.6	
−14.6 (< Ring)		Average diff.	−11.0 (< Ring)		
4 + Ring, 1 + Donor			4 + Ring, 1 + Donor		

Since 5 of 6 animals tested revealed that S^{35} uptake was within the experimental error in both left and right corneal center disk (as indicated by the italicized figures in TABLE 1), it was thought desirable to express S^{35} remaining in the cornea in the hot donor-cold host experiment instead of cpm/dry weight. This was preferred because of a slight increase in dry weights 7 and 14 days after surgery, probably due to fibrin adhesion and an ingrowth of granulation tissue. The 5-mm.-center disk stored at −15° C., which indicated approximately the amount of incorporated S^{35} present in the donor disk at the time of surgery, was compared to the transplanted disk.

Hot donor-cold host. The average percentages of S^{35} remaining in fresh transplanted cornea for 7 and 14 days were 66.7 per cent (TABLE 2) and 40.6 per cent (TABLE 3), respectively. It is also interesting to note the average percentages remaining for formalin-treated, 12 P, and 12 NP after 7 days were 69.5, 68.2, and 65.2, respectively (TABLE 2). Fourteen days after surgery the average percentages remaining for formalin, 12 P, and 12 NP were 35.8, 41.1, and 37.8, respectively. Thus no significant differences among groups were noted. The elimination rates did not appear to be significantly affected by the clinical state or even by slight vascularization of the graft.

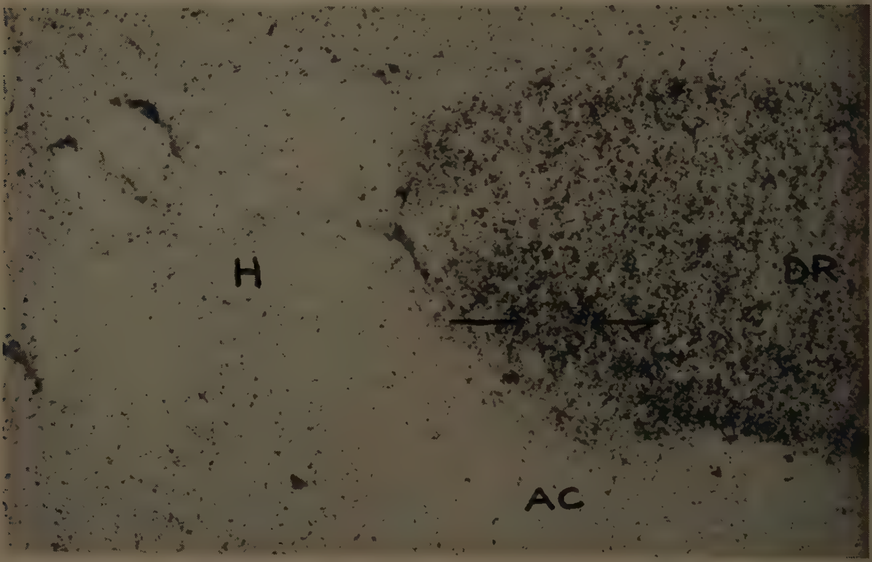

FIGURE 2. S^{35}-incorporated freshly transplanted donor 7 days after surgery. Note the concentration of S^{35}-positive material toward the posterior of the cornea at the wound edge (*arrows*). Donor (DR) still positive 7 days after surgery. Host (H) shows no significant uptake of S^{35}. Anterior chamber (AC). Radioautograph type NS, exposure 36 days. ×90.

Seven and 14 days after surgery the radioautographs revealed in each group a concentration of S^{35}-positive material still within the donor cornea and in the host's cornea at the wound edge. However, beyond the wound edge within the unlabeled host, uptake of S^{35} significantly above background could not be distinguished. Thus it would appear that most of the bound S^{35} 14 days after surgery has been either eliminated into the aqueous humor and/or tears, or dissolved and carried away from the host during histological preparation.

Hot host-cold donor. Animals were labeled with S^{35}. Three days later when most of the inorganic S^{35}-sulfate was excreted and a small fraction incorporated into sulfated mucopolysaccharides[3,4,7] a cold-donor cornea was transplanted to a hot host. The S^{35}-labeled incorporated material was observed radioautographically and quantitatively entering the previously cold-donor cornea. The ring (10 mm.) of the host, which was labeled along with the host animal, and the donor (5 mm.) corneal disk were compared and the readings expressed as cpm per dry weight tissue. Seven days after surgery, as indicated in TABLE 4, S^{35}-positive material had entered the graft, but it was not evident in any of the 4 groups whether the donor or the ring gave the greater number of impulses per dry weight. This was the case because of the large variation among the members of each group. Thus a statistically accurate relationship could not be established between host and donor tissues. Fourteen days after surgery, however, a trend was noticeable (TABLE 5). Of a total 18 donors and 18 rings quantitated for S^{35}-sulfate in fresh, 12 P, and 12 NP, all but 3 cases indicated that the ring had more counts per dry weight of corneal tissue. On the other hand, for formalin-treated cornea, a total of 8 quantitations each for ring and donor cornea, all but 1 indicated that the donor had more counts per dry weight of tissue. The average cpm per dry weight for fresh, formalin-treated, 12 P, and 12 NP donor corneas were 42.5, 70.0, 32.0, and 28.6, respectively; for rings they were 55.1, 41.1, 46.6, and 39.6, respectively. The median values for cpm per dry weight are as follows: fresh donor, 26; ring, 49; formalin-treated donor, 59; ring, 43; 12 P donor, 22; ring, 44; 12 NP donor, 20; and ring, 42. Thus 14 days after surgery formalin-treated donors indicate an average of 28.9 addi-

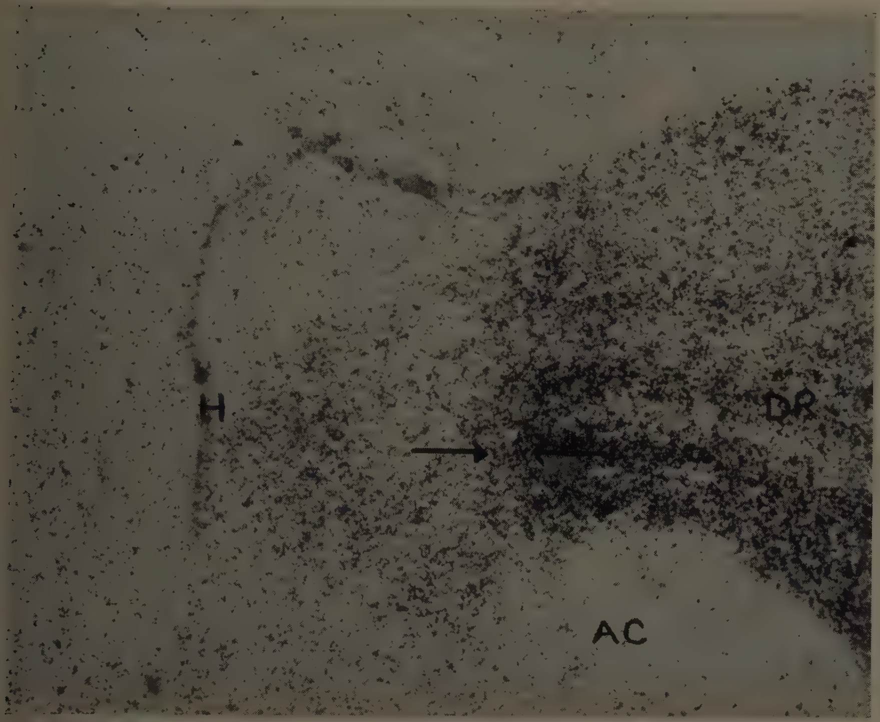

FIGURE 3. S^{35}-incorporated formalin-treated donor cornea 14 days after surgery. Donor (DR) still positive 14 days after surgery, particularly toward the anterior chamber (AC) at the wound edge (*arrows*). Note positive host (H) toward the anterior chamber at the wound edge. Radioautograph type NS, exposure 35 days. ×90.

tional cpm per dry weight tissue than do the rings of its host. Fresh, 12 P, and 12 NP donor 5-mm. disks indicate an average of 12.6, 14.6, and 11.0 less cpm per dry weight than does its host's ring (FIGURE 1). No significant differences were noted, however, between fresh, 12 P, and 12 NP rings.

Radioautographs, as indicated in FIGURES 2 to 8, show prelabeled S^{35}-sulfated mucopolysaccharides 7 and 14 days after surgery within wound edges and more or less evenly distributed throughout the previously unlabeled donor. Thus there are indications that at least a part of the ground substance is replaced within 14 days after surgery.

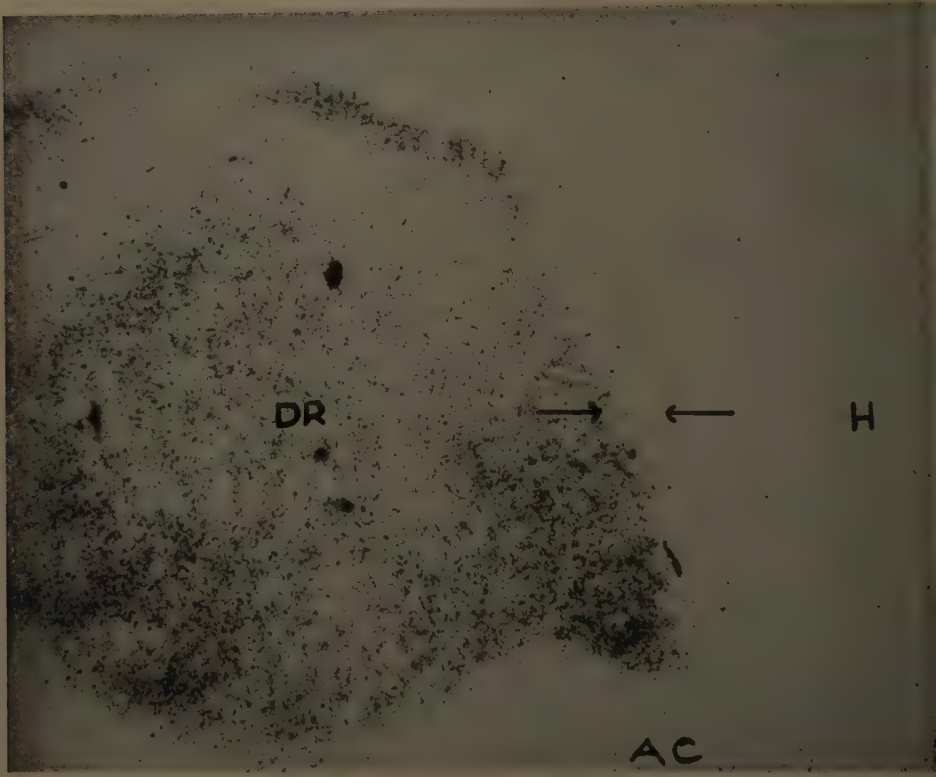

FIGURE 4. S^{35}-incorporated donor transplanted cornea 7 days after surgery. Cornea was preserved in PVP and stored for 12 days prior to surgery. Note positive S^{35}-donor cornea (DR) and S^{35}-negative host (H) cornea except at wound edge (*arrows*). Anterior chamber (AC). Radioautograph type A, exposure 40 days. ×90.

Histology. Alcian blue and periodic acid-Schiff (PAS) stains indicated no difference in staining qualities between the host and donor corneas. Toluidine blue O slides gave a well-defined reddish-pink color along the wound edge. Thus a possible concentration of acid mucopolysaccharides within that area may be present. Many fibroblasts were noted at the wound edge both 7 and

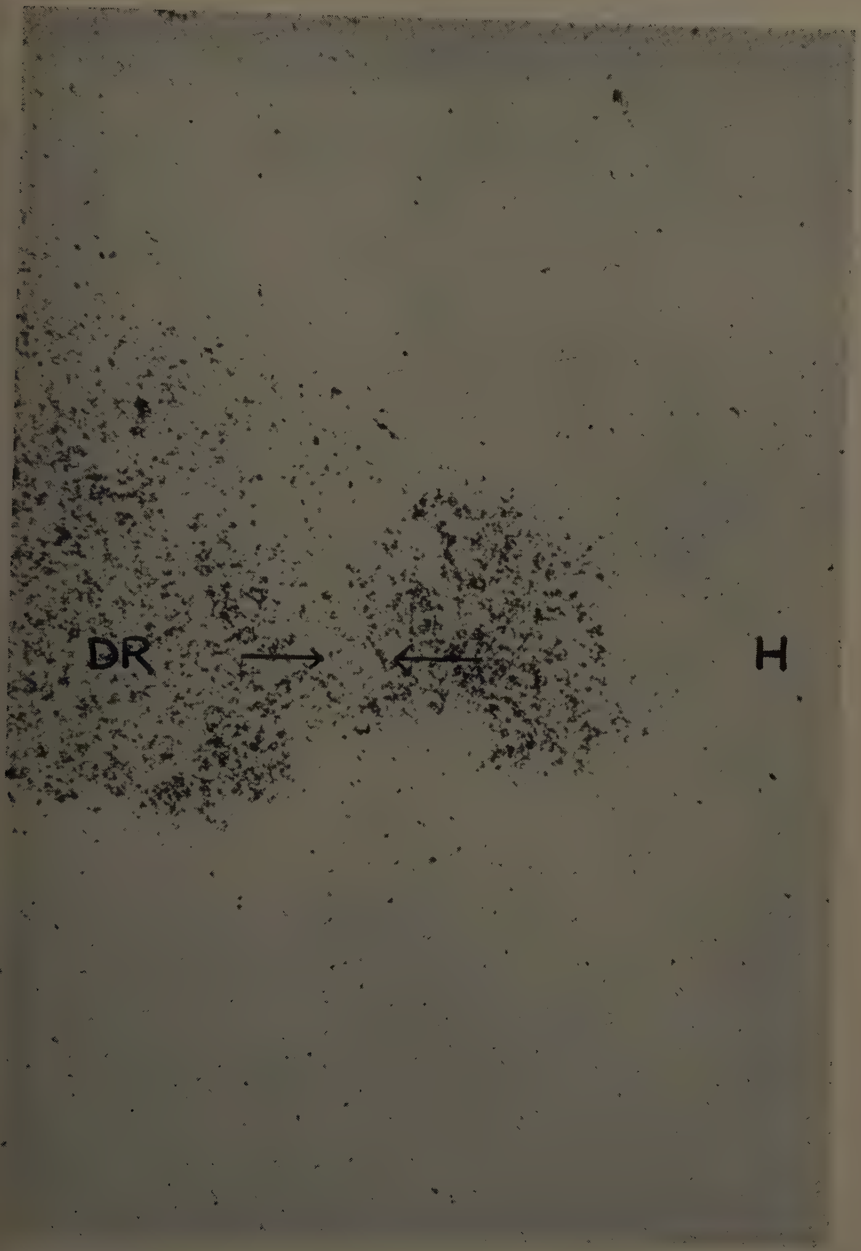

FIGURE 5. S^{35}-incorporated transplanted donor cornea 14 days after surgery. Cornea was preserved in PVP and stored for 12 days prior to surgery. Donor (DR) S^{35}-positive, host (H) positive at wound edge (*arrows*). Radioautograph type NS, exposure 35 days. ×90.

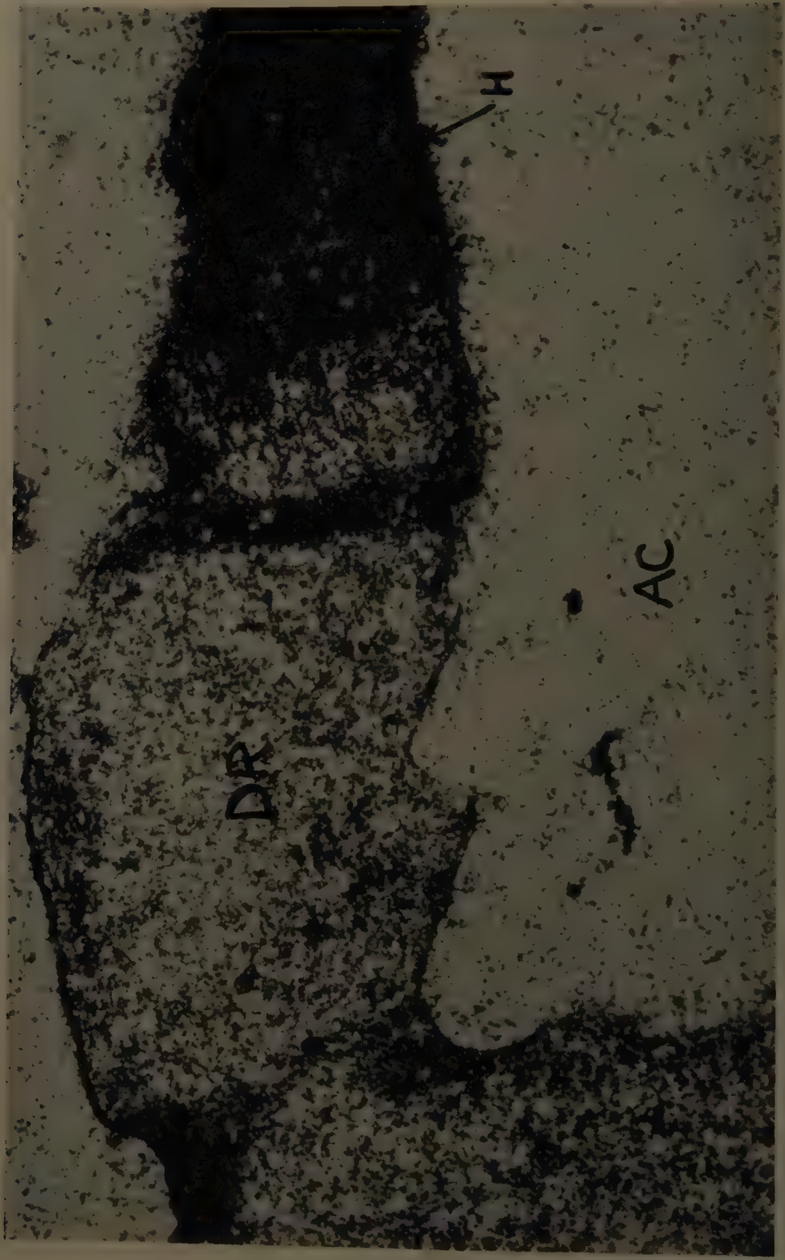

FIGURE 6. Nonradioactive fresh donor (DR) transplanted to an S^{35}-positive host (H) 7 days after surgery. Note the S^{35}-positive donor cornea, which had been cold prior to surgery. Anterior chamber (AC). Radioautograph type NS, exposure 35 days. ×90.

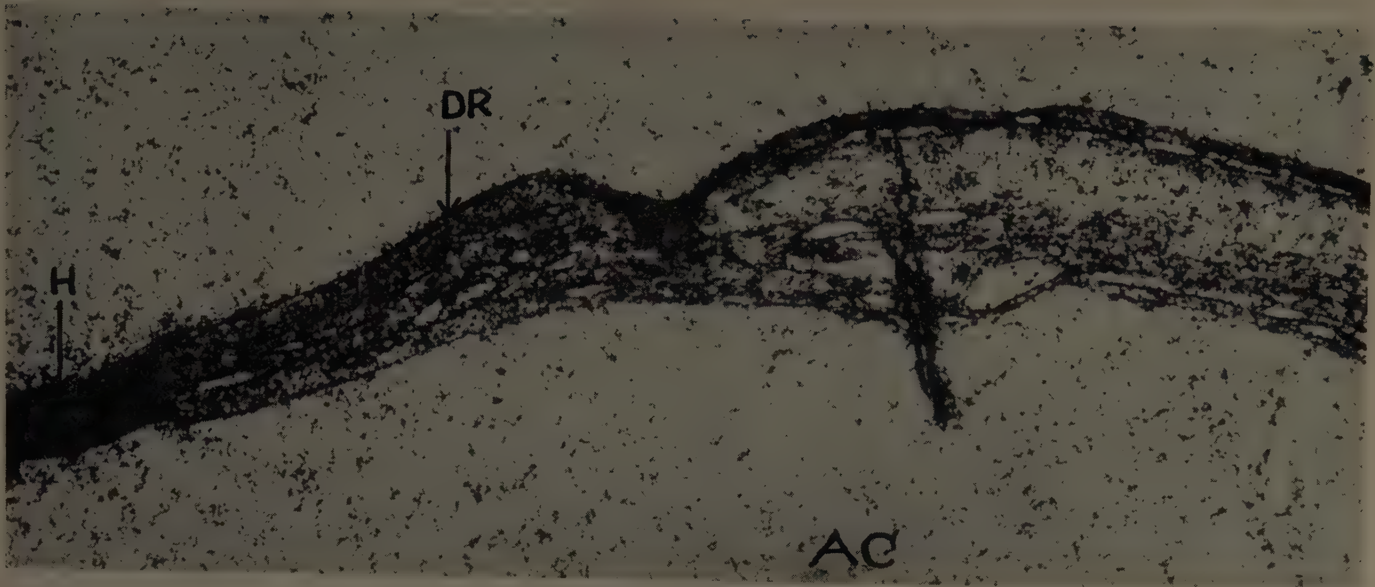

FIGURE 7. Nonradioactive fresh donor (DR) cornea transplanted to an S^{35}-positive host (H) 14 days after surgery. Note the formerly cold S^{35}-positive donor cornea. Anterior chamber (AC). Radioautograph type NS, exposure 34 days. ×90.

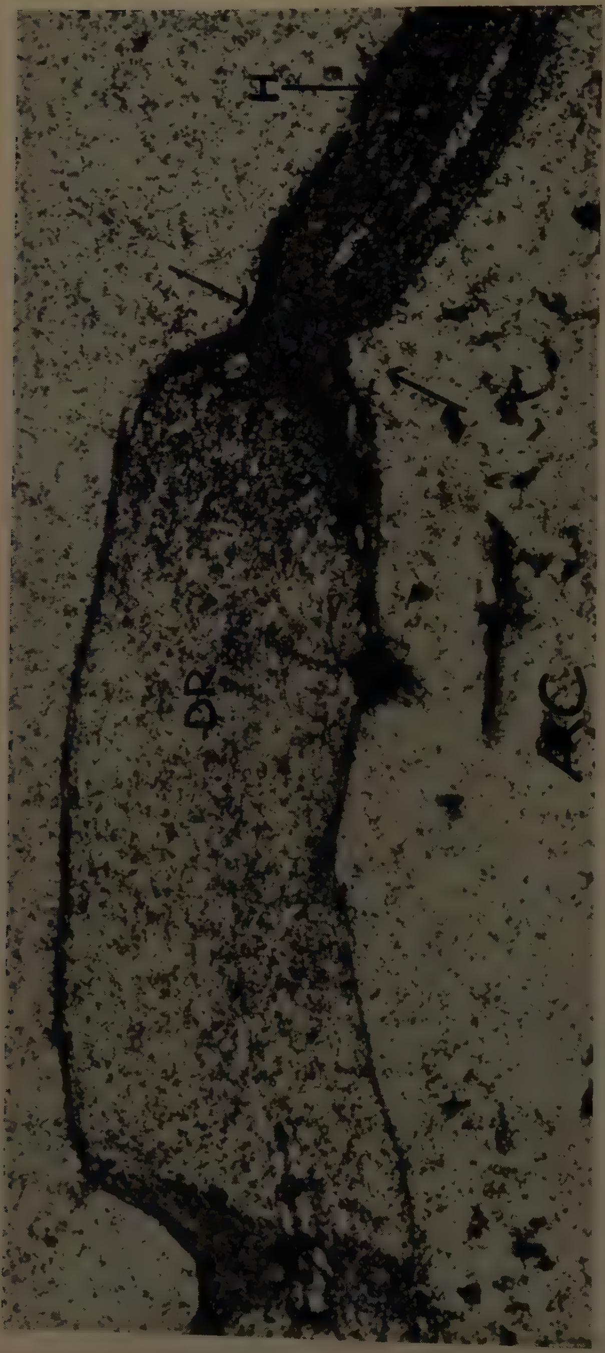

FIGURE 8. Nonradioactive formalin-treated donor cornea transplanted to a radioactive host (H) 14 days after surgery. Note the S^{35}-positive formerly cold donor (DR) with a heavy concentration near the wound edge (*arrows*). Anterior chamber (AC). Radioautograph type NS, exposure 34 days. ×90.

14 days after surgery. A fibrin clot filled the wound. The epithelium was completely regenerated on the trephine cut by the seventh day. In some cases, when host and donor corneas lacked good apposition, the epithelium dipped into the wound. Sections showed no histological evidence of destruction of the stromal fibers or of Descemet's membrane.

Discussion

It is interesting to note that in the normal rabbit 18 per cent more S^{35}-sulfated mucopolysaccharides are taken up by the central area of the cornea than by the periphery, as determined 3 days after administration of the isotope. Two different zones seem to exist in the cornea, central and peripheral, as demonstrated by toluidine-blue stain carried out by Pratt-Johnson.[14] A narrow pale-purple peripheral and a navy-blue central area were observed. This may constitute a histochemical correlate to our findings, pointing toward a mucopolysaccharide gradient with a greater concentration in the center of the cornea. It seems of some significance that an apparently homogeneous tissue such as the cornea should exhibit such a gradient, which may or may not be due to the distance from the nearest vascular bed. It may be entirely possible that this gradient may be the result of turned-over labeled molecules that move toward the center of the cornea and then are reincorporated by keratoblasts, thus giving rise to the zonal differences quantitatively.

For transplanted fresh labeled donor cornea, the average percentages of S^{35}-sulfate remaining in the donor 7 and 14 days after surgery were found to be approximately 66 per cent and 40 per cent, respectively. The average percentages remaining in the donor disks of the other 3 groups 7 and 14 days after surgery were not found to be significantly different from that of fresh transplanted tissue. The clinical state of the graft did not influence the rates of elimination quoted above. It seems worth mentioning in this connection that in some cases even a considerable degree of vascularization of the graft did not alter the rates of S^{35} elimination in any of the 4 experimental groups.

Seven and 14 days after surgery radioautographs revealed a concentration of S^{35}-positive material still within the donor and in the host at the wound edge. This was true for each of the groups. However, beyond the wound edge in the unlabeled host, nothing significantly positive was indicated over background. Thus it would appear that 2 weeks after surgery most of the approximately 66 per cent of bound S^{35} had been eliminated via the aqueous humor or the tears, or both. However, elimination through the host may have occurred also. Due to a possible enzymatic breakdown of the mucopolysaccharides, histological procedures may have dissolved and carried away any of the S^{35} previously taken up by the host. It seems more likely that that the escape of ground substance had occurred via the aqueous humor rather than because of the histological technique used, since a flow of S^{35}-sulfate to the donor does occur. This is also borne out by radioautographs that indicate at the wound edge of the host a greater density of S^{35}-positive material toward the anterior chamber.

Dohlman[15] observed respiration rates and uptake of S^{35} into bovine corneas preserved by various methods. He noted that after treatment with 5 per cent formalin, whether for one half hour or for a few seconds, respiration and S^{35} uptake ceased completely. In addition, cornea treated with formalin prior to surgery generally is accepted by the host and usually gives poor clinical results. In our unpublished experiments cornea preserved with PVP and stored for 12 days has yielded approximately 15 per cent more clear donors after 6 weeks than nontreated cornea stored for the same period. We may assume safely that the elimination rate of S^{35}-sulfated mucopolysaccharides is not affected by the state of the graft prior to surgery: that is, despite the usually good clinical results expected, the respiration rate, and state of hydration of the corneal tissue at the time of keratoplasty.

Seven days after surgery part of the donor's ground substance was replaced by the host, as depicted in the radioautographs (FIGURES 2 to 8). It is puzzling that quantitative data indicated no noticeable trend in any of the 4 groups as to whether donor or ring had more S^{35} per unit of dry weight. In addition, it is also difficult to explain why such a large variation of S^{35} uptake exists among the members of each group.

Fourteen days after keratoplasty unlabeled formalinized donor disks were found to contain more S^{35}/dry wt. than did the corneas of their host. On the other hand, in each of the other groups the reverse situation was true and, pending further investigation, the reasons for this occurrence necessarily must be conjectured. It is conceivable that this occurrence may be due to the following reason: after the drastic changes that the donor tissue underwent during formalin fixation, the natural barriers of the donor were destroyed and allowed for a nonselective infiltration by substances from the host, including its ground substance. The donor tissue then may have acted as a sponge.

Radioautographs 7 and 14 days after surgery show that S^{35}-sulfated mucopolysaccharides of the ground substance enter the donor grafts; thus the ground substance is replaced, at least in part. Since the ground substance is usually produced by the keratoblasts, its ultimate fate could not be determined in this experiment, as the fate of the keratoblasts is not understood. However, if keratoblasts are replaced immediately or soon after surgery, then the ground substance necessarily would be replaced shortly after keratoplasty.

Summary

An average of approximately 18 per cent more S^{35}-incorporated material was found to be present (control rabbits) in the 5-mm.-center trephined disk as compared to its immediate 10-mm. peripheral cornea.

The percentage of S^{35} remaining in a donor graft when transplanted to an unlabeled animal was determined. In the graft there remained approximately 66 per cent after 7 days and 40 per cent after 14 days. No significant difference was noted between formalin-treated cornea, cornea preserved in PVP and stored twelve days (12 P), and nontreated cornea stored for 12 days (12 NP).

Radioautographs of each group indicated no significant elimination of S^{35} through the cornea of the host.

Animals labeled with S^{35} indicated 14 days after surgery that more S^{35} had entered the previously unlabeled formalin-treated donor than fresh, 12 P, and 12 NP transplanted cornea.

Radioautographs indicate that S^{35}-sulfated mucopolysaccharides of the ground substance are replaced by the seventh day and are still evident by the fourteenth day after surgery.

Sections stained with toluidine blue gave a well-defined metachromatic reddish-pink color along the wound region.

Acknowledgment

I express my deep appreciation to Louis L. Bergmann for his interest and support.

References

1. Dohlman, C. H. 1957. On the fate of the corneal graft. Acta Ophthalmol. **35:** 286.
2. Dohlman, C. H. 1957. On the metabolism of the corneal graft. Acta Ophthalmol. **35:** 303.
3. Dziewiatkowski, D. D. 1949. Rate of excretion of radioactive sulphur and its concentrations in some tissues of the rat after intraperitoneal administration of labeled Na_2SO_4. J. Biol. Chem. **178:** 197.
4. Dziewiatkowski, D. D. 1951. Isolation of chondroitin sulfate-S^{35} from cartilage of rats. J. Biol. Chem. **189:** 187.
5. Meyer, K. & E. Chaffee. 1940. The mucopolysaccharide acid of the cornea and its enzymatic hydrolysis. Am. J. Ophthalmol., **23:** 1320.
6. Meyer, K., A. Linker, E. Davidson & W. Weissman. 1953. The mucopolysaccharides of bovine cornea. J. Biol. Chem. **205:** 611.
7. Dohlman, C. H. 1957. Incorporation of radioactive sulfate into the rabbit eye. Acta Ophthalmol. **35:** 115.
8. Nathaniel, A. & R. Z. Levene. 1959. Mucopolysaccharide sulfate in the cornea and sclera of rabbits. Arch. Ophthalmol. **61:** 641.
9. Paton, R. T. 1955. Keratoplasty. : 137. McGraw-Hill. New York, N. Y.
10. Stocker, F., A. Eiring, R. Georgiade & N. Georgiade. 1958. Evaluation of viability of donor tissue for corneal grafting. Trans. Am. Ophthalmol. Soc. **56:** 217.
11. Moltke, E. 1957. Uptake of S^{35}-sulfate by healing wounds: effect of thyroxine and of ascorbic acid deficiency. Acta Endocrinol. **25:** 179.
12. Manual of Histologic and Special Staining Technics by Armed Forces. 1957. Inst. Pathol., Washington, D. C.
13. Simmel, E. B., P. J. Fitzgerald & J. T. Godwin. 1951. Staining of radioautographs with metanil yellow and iron hematoxylin. Stain Technol. **26:** 25.
14. Pratt-Johnson, J. 1959. Studies on the anatomy and pathology of the peripheral cornea. Am. J. Ophthalmol. **47:** 478.
15. Dohlman, C. H. 1956. The viability of the stored cornea. Acta Ophthalmol. **34:** 218.

TRANSPLANTESE

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It might be supposed that anyone criticizing transplantese terminology must have exceptional knowledge of the classics. This is certainly not true in my case. In certain respects I have been driven into this position. I first used the terms isoantigen and isoantibody in connection with tumor transplantation in 1937. No one objected at the time, but there have been numerous complaints since then. I have been asked to substitute other terms, called upon to give a public explanation of the use of "isoantibodies" in the "homologous situation" and, finally, I have been accused of introducing the terms subsequent to Snell's introduction of the term isograft.[1] I must inform such critics that they do not enhance their reputations by such remarks. Such terms as isoantigen and isoantibody, have been in use about fifty-three years longer than isograft. Common sense alone should suggest that it is much easier to change a seven-year-old term than one sixty years old. Quite apart from this, such people show an extraordinary arrogance. We are simply a branch of immunology, and we should try to adapt our terminology to suit the general body of immunologists, rather than the reverse.

I suppose I should be very flattered to have been thought the inventor of "isoantibody" since, in fact, it was the great Paul Ehrlich who first used the term. The year 1900 was very important for our subject. Landsteiner published his first paper on the human blood groups.[2] Ehrlich and Morgenroth published their third communication on hemolysines.[3] They exchanged blood between goats and found that the resulting sera would lyse cells of other goats, but not those of the animal supplying the serum. They called the antibodies "isolysines." Literally translated, this means equal lysins. We are not told why they did not call the antibodies homolysins, which would mean same lysins, but I think we can make a fairly good guess. One obvious reason is that homolysin and hemolysin are much too similar. However, it is possible that they had an additional end in view. What we now call isolysis, in fact, had been observed previously, but the phenomenon was regarded as entirely pathological, and the antibodies were confused with autolysins. Ehrlich and Morgenroth in 1900 emphasized the fact that the antibodies studied by them were *not* autolysins. The word homolysin could be interpreted as same individual lysine whereas this was hardly likely with isolysin. Landsteiner used the word isoagglutinin for the first time in the next year, and it is easy to see how such general terms as isoantibody and isoantigens came to be accepted.

The word isograft has an entirely different history, being derived from the geneticists' term isogenic. I asked Snell, who introduced this term, when he did so. He was unable to tell me, but I understand it was in use in his student days. We may ask why the term "genetically equal" was used for genetic uniformity when the word homogeneous was already there? Here again I must guess. If you look up the work homogenetic in a dictionary, you will find that it denotes community of descent, a rather vague term that might denote varying degrees of relationship. The word heterogenetic is, of course, its opposite. Until well after the time of Louis Pasteur the advocates of the term "heterogenesis" were those who believed, for example, that eels were generated from grass or molds by moisture; it will be seen, therefore, that there were good reasons for avoiding "homogenetic" and heterogenetic." Isograft, accordingly, is an abbreviation of isogenic graft. I have made the suggestion that "isogenic graft" should be used in place of "isograft." I now think this is a mistake. The correct expansion of "isograft" is "isogenic *homograft*."

I am afraid this must seem blasphemous to loyal transplantesians but, before they explode, may I ask them to consider the literal meaning of our totem words. Such terms as homograft and homiotransplant mean simply "same graft" or "same transplant." These terms have been used to mean "same person graft" or autograft, but now all workers in the field accept the convention that it means "same species graft." If an A strain mouse is of the same species as a CBA mouse, surely two A-strain mice or two CBA mice are members of the same species, and intrastrain grafts are therefore a special sort of intraspecific graft. However, for about the last five years we have been saying that, if two individuals are genetically identical, grafts exchanged between them are *equal*, but *not* the same. On the other hand, if they are genetically different, their grafts *are* the *same*, but *not* equal. It is here that transplantese ceases to be homologous with English or indeed with common sense.

This situation reaches its full flowering with that dismal trio isologous, homologous, and heterologous. I suppose that these terms must have some great appeal by virtue of their sound because there is no reason for using any of these words in their present context. The correct technical term for the isologous situation is clearly isogenic, but I suppose if people think isologous sounds more imposing they should be allowed to use it.

Such people do not have the same freedom with regard to homologous, since no scientist or group of scientists, however eminent, has the right to attach a technical meaning to a word that directly contradicts any of its meanings in general use. The word homologous can be given several shades of meaning but, in general, we say things are homologous if they show some sort of agreement or correspondence. The type of bone marrow that agrees most closely with an A-strain mouse is an A-strain marrow that is therefore the homologous marrow. Prior to the introduction of transplantese we often referred to an intrastrain graft as a graft within the homologous pure line. If we transferred tissue to a foreign strain, we often referred to it as a heterologous pure line. No one objected, and no one had any grounds for objection, as the use of homologous and heterologous in this context is perfectly correct. In our present Mad-Tea-Party jargon we describe violent disagreement between graft and host as "homologous disease!"

Immunologists often use the words homologous and heterologous. Sometimes the terms refer to homologous or heterologous species, but not necessarily. In general, we regard the homologous situation as that in which antibodies react with the antigens used to elicit them. Suppose one were to make synthetic antigens by coupling the three isomers of tartaric acid to a protein. One would probably find that, even if strong reactions were found with the homologous isomers, those obtained with either of the heterologous isomers were relatively weak. To bring the argument nearer home, I quote from a typescript I read recently. The author was discussing the detection of tumor-specific antigens if inbred strains are not available. The author said: "The only really adequate controls appear to be normal homologous cells from the individual in which the tumour arose." I did not demand an explanation for his use of "homologous in the autologous situation" because his statement is correct and comprehensible to anybody but a transplantesian. I am likely to run into trouble myself soon. I hope to write a paper shortly upon what D. B. Amos and I called the X antigens of leukemic cells. These are recognized by passive immunity experiments in the homologous pure line—I beg your pardon—in the isologous situation. The results are rather similar to those I mentioned concerning the isomers of tartaric acid. The easiest way to express this is to say that, with one rather interesting exception, protection is always most effective with the homologous leukemia. I may try to avoid using heterologous, but I shall find it very difficult to avoid using homologous in its correct sense, and I see no reason why I should do so.

There can be no doubt that isogenic is a more accurate term than isologous. I do not like heterologous, not because it is wrong, but because heterospecific is more definite. It seems clear that we badly need an antithesis to isogenic in our vocabulary. I have asked several classical scholars for their advice and have received a number of suggestions. The term I liked best was "allogenic," which is derived from the Greek *allos* meaning "other." If this is agreed upon we should then have isogenic, allogenic, and heterospecific instead of isologous *et al.*, whose burial is long overdue.

The prerequisite for the peaceful coexistence of isograft and isoantibody is, as George Snell once put it, that people should study their verbal tools. However, I think it might be a very good idea if some writers were to introduce "isogenic homograft" into their papers to help educate newcomers, especially those who are not geneticists and may not know the term isogenic. I am more worried about the antithetic situation. We cannot contemplate retaining the simple term homograft as an antithesis to isograft, since this is really the cause of all our verbal troubles. However, in situations where inbred strains are not available or are not being used, the type of homograft will be obvious from the context. Similarly, we need not worry about homograft reactions since they do not occur with isogenic homografts. However, when we are contrasting isogenic homografts with allogenic homografts, I think the latter term should be used. It may be left to individual judgment whether one abbreviates to "allograft." If this is done, I hope it will not be done too quickly; people must become accustomed to the fully expanded form. We should have saved ourselves a lot of trouble had we done so with "isograft."

References

1. Snell, G. D. 1958. Transplantable tumors. *In* The Physiopathology of Cancer. : 293–345. 2nd ed. F. Homburger, Ed. Hoeber. New York, N. Y.
2. Ehrlich, P. & J. Morgenroth. 1900. Ueber Haemolysine. Dritte Mittheilung. Berlin. klin Wochschr. **37:** 453–458.
3. Landsteiner, K. 1901. Ueber Agglutinationerscheinungen normalen menschlichen Blutes. Wien. klin. Wochschr. **14:** 1132–1134.

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